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ACTIVATION OF HUMAN NATURAL KILLER CELLS BY *Plasmodium falciparum*

Katerina Artavanis-Tsakonas

**Thesis submitted to the University of London in fulfillment of the requirements for
the Doctorate of Philosophy**

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**Immunology Unit
Department of Infectious and Tropical Diseases
London School of Hygiene & Tropical Medicine
Keppel Street
London
WC1E 7HT**

Abstract

The purpose of work described in this thesis was to (i) determine the contribution of innate immune responses to the early pro-inflammatory cytokine response to *Plasmodium falciparum*, (ii) describe the kinetics and cellular sources of IFN- γ production by human PBMC in response to activation by intact, infected erythrocytes (iRBC) or freeze-thawed schizont lysate (PfSL) and (iii) determine the activation requirements for innate immune cells responding to *P. falciparum*. Infected erythrocytes induce a more rapid and intense IFN- γ response from malaria naïve PBMC than does PfSL, correlating with rapid iRBC activation of CD3-CD56+ natural killer (NK) cells to produce IFN- γ . There is marked heterogeneity between donors in the magnitude of the NK-IFN- γ response not correlating with mitogen or cytokine-induced NK activation or prior malaria exposure. The NK-IFN- γ response is highly IL-12 dependent, partly IL-18 dependent and highly dependent on direct contact between the NK cell and the parasitized erythrocyte. Exogenous rIL-12 or rIL-18 did not augment NK-IFN- γ responses indicating that IL-12 and IL-18 production is not the limiting factor explaining differences in NK cell reactivity between live and dead parasites or between donors. The possibility that donor heterogeneity is due to genetic variation in killer immunoglobulin-like receptors (KIR) and/or differential expression of C-type lectin receptors was also investigated. A significant up-regulation of CD94 and NKG2A was observed in IFN- γ + NK cells of responding donors, suggesting that the inhibitory CD94:NKG2A heterodimer may serve a regulatory function on *P. falciparum* activated NK cells. Collectively, these data indicate that NK cells may represent an important early source of IFN- γ , a cytokine implicated in induction of various anti-parasitic effector mechanisms. The heterogeneity of this early IFN- γ response between donors suggests variation in their ability to mount a rapid pro-inflammatory cytokine response to malaria that may, in turn, influence their innate susceptibility to malaria infection, malaria-related morbidity or death from malaria.

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List of Abbreviations

AB	human AB serum
$\alpha\beta$	alpha-beta T cell receptor
APC	allophycocyanin
BSA	bovine serum albumin
CD	cluster of differentiation
CMV	cytomegalovirus
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
$\gamma\delta$	gamma-delta T cell receptor
GM	growth medium
GPI	glycosyl-phosphatidylinositol
HBSS	Hanks Balanced Salt Solution
HIV	Human Immuno-deficiency Virus
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule-1
IL	Interleukin
IFN	Interferon
Ig	immunoglobulin
iRBC	infected red blood cells
LSHTM	London School of Tropical Medicine & Hygiene
MAb	monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MSP	merozoite surface protein
NK	natural killer
NO	nitric oxide
OPD	o-phnyleneiamine
PBL	peripheral blood lymphocytes
PBMC	peripheral mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PfSL	<i>P. falciparum</i> schizont lysate
PHA	phytohemagglutinin
PPD	purified protein derivative
RBC	red blood cells
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulphate
SSC	side scatter
TCR	T cell receptor
TNF	Tumor necrosis factor
TGF	transforming growth factor
uRBC	uninfected red blood cells

Chapter 1 Introduction

1.1 Malaria- the problem

Throughout history malaria has proved to be a significant health threat to humans. Along with tuberculosis and HIV, it is one of the biggest causes of infectious disease deaths in the world today and endangers 2,200 million people, i.e. 40% of the world's population. Over 90% of the morbidity and the mortality of this disease occurs in the Sub-Saharan region of the African continent (Sachs *et al.*, 2002), with the majority of the remaining cases in areas of Southeast Asia and South America (Figure 1.1).

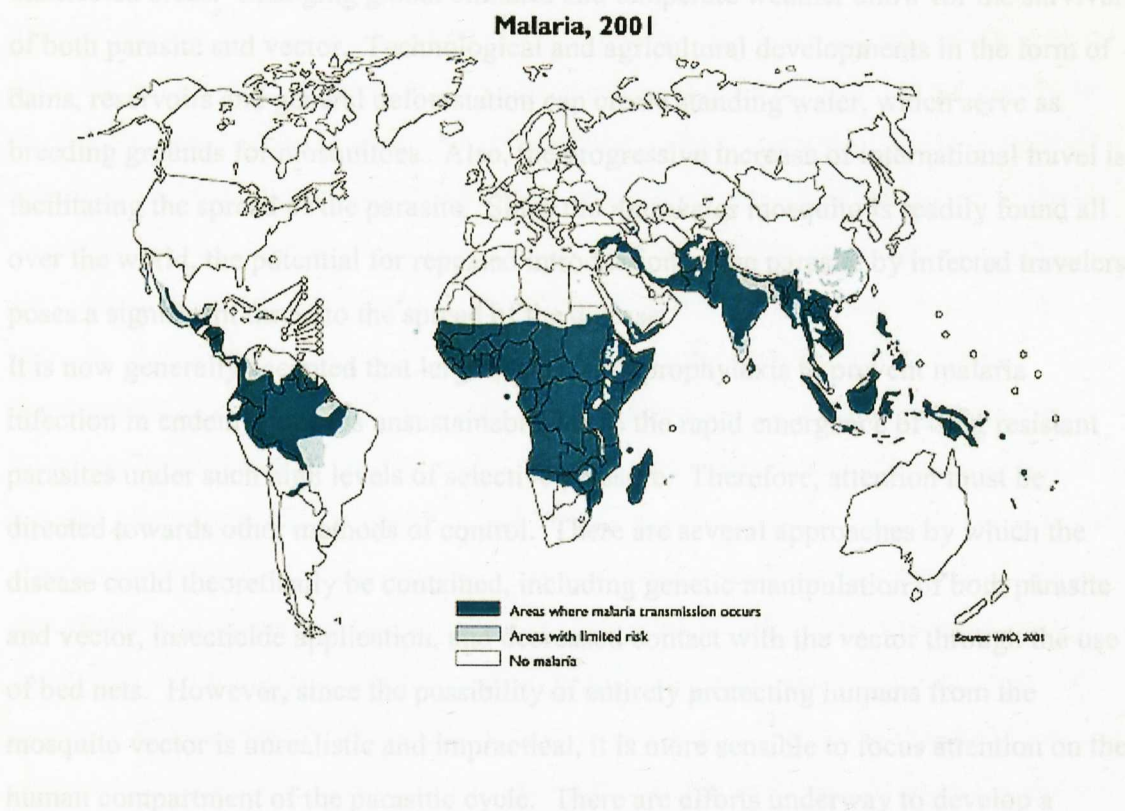


Figure 1.1: World distribution of *P. falciparum* malaria. This map outlines the presence of malaria throughout the world as determined by the WHO in 2001. Areas in dark green correspond to regions of stable transmission, areas in light green to regions of low or unstable transmission and areas in white to malaria-free regions.

Between 300-500 million clinical cases occur each year worldwide, approximately 2 million of which are fatal, primarily in children. The economic repercussions of the disease are also immense, with malaria-endemicity being associated with poverty and reduced rates of economic growth as compared with non-malarious regions (Sachs *et al.*, 2002). This correlation is not surprising considering that aside from the high chance of untimely death, survivors of this disease are often left with permanent disabilities such as neurological defects, taking them permanently out of the workforce or reducing their educational achievements (Greenwood *et al.*, 2002).

Due to environmental and technological changes throughout the globe, malaria is beginning to threaten the developed world as well, and is intruding into new, previously unaffected areas. Changing global climates and temperate weather allow for the survival of both parasite and vector. Technological and agricultural developments in the form of dams, reservoirs and general deforestation can create standing water, which serve as breeding grounds for mosquitoes. Also, the progressive increase of international travel is facilitating the spread of the parasite. Since the *Anopheles* mosquito is readily found all over the world, the potential for repeated introduction of the parasite by infected travelers poses a significant threat to the spread of the disease.

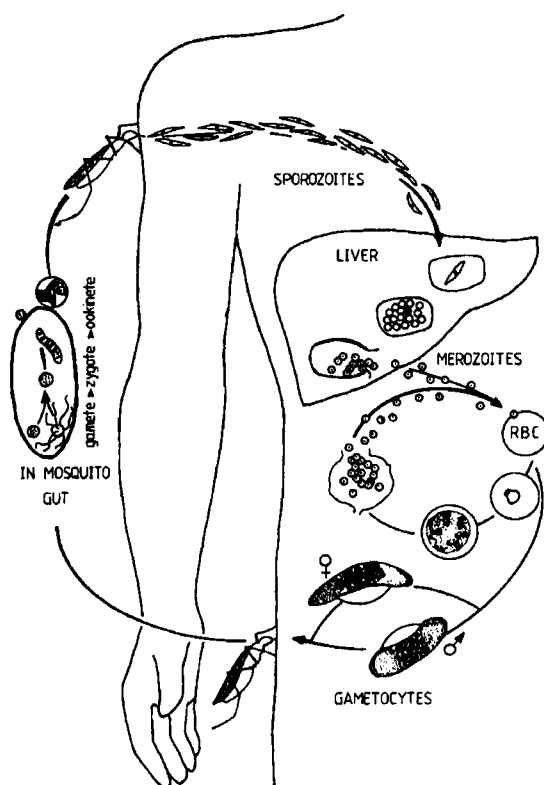
It is now generally accepted that large scale chemoprophylaxis to prevent malaria infection in endemic areas is unsustainable due to the rapid emergence of drug resistant parasites under such high levels of selective pressure. Therefore, attention must be directed towards other methods of control. There are several approaches by which the disease could theoretically be contained, including genetic manipulation of both parasite and vector, insecticide application, and decreased contact with the vector through the use of bed nets. However, since the possibility of entirely protecting humans from the mosquito vector is unrealistic and impractical, it is more sensible to focus attention on the human compartment of the parasitic cycle. There are efforts underway to develop a vaccine using rationale vaccine design since so far, empirical attempts have failed. This approach requires a better understanding of the host-pathogen interactions at each stage of infection.

1.2 Malaria- the parasite

Plasmodium parasites have the ability to multiply themselves by a factor of approximately 10 about every two to three days. Of the four types of *Plasmodium* that infect humans, namely *P. ovale*, *P. malariae*, *P. vivax* and *P. falciparum*, our research focuses on *P. falciparum* as it is the most virulent and pathogenic of the four and accounts for the vast majority of malaria cases and almost all malaria-related deaths. Figure 1.2 illustrates the developmental cycle of the parasite.

The sexual phase of the life-cycle takes place in the mosquito and the asexual phase in the human. When an infected mosquito bites, it inserts sporozoites into the bloodstream; sporozoites are small, motile forms of the parasite and are able to find their way to the liver. Once in the liver, the sporozoites infect hepatocytes and undergo asexual division to produce merozoites. Upon release, these merozoites infect red blood cells and begin the blood stage of the infection, consisting of the cyclical maturation into trophozoites and schizonts which eventually burst releasing new merozoites capable of infecting new red blood cells. This periodic rupturing of the red blood cells contributes to anemia and is the phase of the life cycle where acute symptoms of malaria occur. Certain merozoites differentiate into gametocytes, which are the sexual stage of the parasite. It is unclear what signals a parasite needs in order to commit to sexual development although several factors have been shown to affect gametocytogenesis, including host immunity, inter-parasite signaling, transmission, and antimalarial drugs. It has also been suggested that variable numbers of merozoites are pre-committed to sexual development, with their ultimate gametocyte conversion dependent on any number and combination of the aforementioned factors (Dyer *et al.*, 2000). Once a parasite has committed to the sexual pathway, the male and female gametocyte are taken up by the mosquito in a blood meal where they emerge from the red blood cell and discard the erythrocyte membrane. Fertilization occurs within the mosquito's gut, when the male microgametocytes penetrate the female macrogametocytes to form zygotes, which in turn develop into oocysts within the stomach wall. Mature oocysts can give rise to about 1000 sporozoites, which migrate to the mosquito's salivary glands and are reintroduced into the human host with the blood meal (figure 1.2).

From: Good, Berzofsky and Miller (1988) Ann. Rev. Immunol 6 633-688.



The life cycle of human malaria

Figure 1.2: The lifecycle of *Plasmodium*. This figure outlines the sexual and asexual developmental stages of the *Plasmodium* parasite. Each stage is explained in more detail in the text. This figure was taken from Good *et al.* (1988) Ann Rev Immunol 6:663-688.

1.3 Pathology and Pathogenesis

From the moment the human host is infected with the parasites it usually takes between 1 to 3 weeks for symptoms to become apparent or, in other words, for the parasites to reach the circulation and amplify their numbers to pathogenic levels. Malaria can manifest at different severities depending on several factors, including age and immunity of the host. Symptoms can range from a mere febrile illness to anemia, cerebral malaria, multi-organ failure to death.

The different manifestations of disease have been closely correlated to levels of circulating pyrogens and the host's ability to contain their systemic production (Grau *et al.*, 1989b). During schizont rupture, soluble parasite components such as the glycosyl-phosphatidylinositol (GPI) anchors of the merozoite surface proteins are released and are thought to stimulate the release of TNF- α (reviewed by Miller *et al.*, 1994). The release of other pro-inflammatory cytokines such as IFN- γ serve to amplify the production of TNF- α and further the disease. The up-regulation of the intercellular adhesion molecule 1 (ICAM-1) in the cerebral endothelium (Aikawa *et al.*, 1990), occurs as a result of these elevated TNF- α levels. It has been proposed that parasitized cells adhere to ICAM-1 and can therefore sequester in cerebral endothelium and may play a significant role in the onset of cerebral anoxia (Miller *et al.*, 1994), which can lead to coma. The excessive release of oxygen radicals such as nitric oxide (NO) has also been linked to cerebral malaria pathogenesis. It has been suggested that TNF- α -stimulated cerebrovascular endothelial cells release high levels of NO which in turn interfere with neurotransmission and induce coma (Anstey *et al.*, 1996).

The pathology of anemia is more difficult to understand, mainly due to the fact that it is impossible to identify malaria as the sole causative agent in areas where hosts can be infected with a myriad of other pathogens. Malarial anemia can be caused by the rupturing of red blood cells as schizonts burst. Otsuki *et al.* (Otsuki *et al.*, 1999) demonstrated that high levels of IFN- γ and TNF- α can inhibit hematopoiesis, which could also contribute to the anemia. Likewise, high TNF- α to IL-10 ratios have been associated with severe anemia (Kurtzhals *et al.*, 1998), (Othoro *et al.*, 1999).

1.4 Epidemiology and Immunology of Malaria Infections

Susceptibility to malaria has proven to be variable and dependent on a range of epidemiological and genetic factors. The first and obvious risk factor is exposure to the parasite, with the subsequent course of disease and development of immunity being partly dependent on the level of endemicity. However, an individual's age, genetic make-up and immunological maturity are all aspects that can skew symptom manifestations.

A host's immune system will very likely first be challenged with *Plasmodium* during infancy if living in a malarious zone. Infants are characteristically resistant to disease symptoms up to the ages of 4 to 6 months (McGuinness *et al.*, 1998). The mechanism of resistance in this population is currently the subject of extensive research and has yet to be characterized satisfactorily (reviewed in Riley *et al.*, 2001). Suggested explanations for the apparent resistance of infants to malaria include the inhibitory presence of fetal hemoglobin to parasite growth and the potentially insufficient level of dietary p-aminobenzoic acid, an essential nutrient for parasite growth. The transfer of maternal antibodies during gestation (Simister 1998), and possibly through breastfeeding (Leke *et al.*, 1992), is also thought to play a role in the infant's resistance. However, these points have been disputed and it seems unlikely that any one of them could be solely responsible for protection during infancy (Wagner *et al.*, 1998).

In stark contrast to the benign nature of infection during early infancy, childhood malaria is characterized by severe anemia and by cerebral malaria, both of which can result in death. Anemia tends to occur between birth and 3 years of age, at which point the risk of cerebral malaria becomes a greater threat until about 7 years of age (Marsh 1992). In regions where transmission is seasonal and endemicity is relatively low, cerebral malaria tends to be the dominant complication (Marsh 1992), whereas in highly endemic areas, levels of severe anemia are higher (Lackritz *et al.*, 1992). Although childhood malaria has been, and continues to be extensively studied, the reasons why certain children are affected over others and why some can support high parasite burdens without life-threatening disease, remain largely undetermined (reviewed in Miller *et al.*, 1994). A factor that could, in part, explain these differences is the development of antibodies against parasite glycosyl-phosphatidylinositol anchor molecules (GPI). These

'toxins' are thought to be involved in malaria immunopathology and there is evidence that anti-GPI antibodies increase in both prevalence and concentration as a function of age, with lowest levels found in parasitemic children (de Souza *et al.*, 2002), (Boutlis *et al.*, 2002).

If childhood bouts of the disease are survived, individuals living under conditions of chronic exposure to malaria begin to develop clinical immunity during adolescence with each subsequent infection resulting in decreasing symptomatology. Sterile immunity, meaning the ability to completely prevent development of parasites in the circulation, is believed to be extremely uncommon. A state of clinical immunity is more characteristic whereby the host can still become infected if exposed, but manages to keep parasitemia at a low level and is able to clear the parasite load quickly and effectively. A clinically immune host is also able to circumvent the clinical symptoms almost entirely. This type of immunity is characteristic of areas with perennial transmission where the population is at constant risk of becoming infected. Adults in such areas who have been repeatedly exposed attain this state of clinical immunity where, upon reinfection, they might suffer only a fever and mild flu-like symptoms due to their ability to rapidly control the inflammatory immune response, possibly due to anti-inflammatory cytokines.

However, if a non-immune adult moves into a malarious region the consequences of disease are characteristically more severe and even deadly. Epidemiological evidence shows that when non-immune individuals move into a malaria endemic area, the risk of severe pathology increases with age (reviewed by Riley 1999) with adults being more susceptible to clinical disease than children. Conversely, adults subsequently develop anti-parasitic immunity faster than children (Baird *et al.*, 1998).

Another interesting phenomenon is that T cells derived from naïve adults, when cultured in vitro with malaria antigen, respond in a classical MHC-restricted manner. These cells express a memory phenotype (Fell *et al.*, 1994) and they proliferate and produce IFN- γ in amounts comparable to true malaria memory T-cells (Dick *et al.*, 1996). It has been proposed that naïve hosts are able to mount a severe pathological response to malaria antigens by producing an inflammatory cytokine response originating from CD4+ T-cell clones that are primed by commonly administered vaccines, commonly occurring environmental or commensal organisms such as mycobacteria, or by other pathogens

such as influenza virus (Currier *et al.*, 1992). *Plasmodium* is thought to structurally mimic these antigens thereby soliciting the reaction of memory cells (Riley 1999). As these cross-reactively primed cells are likely to be more common in older individuals, this may explain the high risk of severe disease in non-immune adults compared with non-immune children.

Adults who live in areas of low endemicity and seasonal transmission are categorized as exposed non-immunes, and are at risk of both mild and severe clinical disease. It is generally accepted that this phenomenon is due to the fact that their immune systems have been well primed with the parasite, and therefore mount a strong inflammatory response at reinfection but have yet to develop the regulatory mechanisms required to control the inflammatory response. They manage to clear parasitemia quite effectively, usually experiencing only a few days of flu-like symptoms, but are at risk of developing severe symptoms (Afari *et al.*, 1995).

Pregnant women also fall into a high risk category. Previous research has established significant differences in how malaria is handled by a pregnant versus a non-pregnant woman's immune system. This phenomenon has been attributed to the appearance of new factors such as hormones and proteins, only present during pregnancy, that have immunosuppressive qualities (Vleugels *et al.*, 1987). Furthermore, differences have also been repeatedly documented between infected primigravids (first pregnancies) and multigravids (multiple pregnancies), namely that women who have been pregnant before are at a lower risk for birth complications (Andrews *et al.*, 2002). Some believe that this discrepancy is due to the fact that these immunosuppressive agents are produced at increasingly lower levels with each subsequent pregnancy thereby allowing the woman to control the parasitic infection more effectively (Vleugels *et al.*, 1987).

An alternative hypothesis centers on parasite sequestration in the placenta. Cellular receptor molecules present in humans have been shown to have a high affinity for schizont-infected red blood cells which allows for parasite sequestration in tissues. One such molecule is chondroitin sulfate A (CSA), a glycosaminoglycan extra-cellular matrix component found on the placental epithelium. Placental parasites extracted from pregnant women seem to have a significant affinity for CSA, whereas those taken from non-pregnant individuals do not express this preference (Fried *et al.*, 1996). It has been

suggested that the acquisition of antibodies against the CSA-binding molecules on infected red blood cells abrogates the pathology associated with malaria infection during pregnancy (Ricke *et al.*, 2000), a point supported by the fact that in a hyperendemic area of malaria transmission, increasing levels of these antibodies are correlated with increasing parity. Other work furthers this hypothesis by showing that plasma derived from multigravids is able to block infected red blood cell adhesion to CSA, whereas plasma from primigravids cannot (Fried *et al.*, 1998). By blocking parasite adherence, these antibodies are able to effectively avert sequestration in the placenta and render the parasites unable to evade the immune response by hiding within the placenta (Williams 1996). Lack of sequestration also abrogates placental inflammation and damage, which is a major cause of low birth weight. This information, combined with the different pathologies seen in primigravid and multigravid, all point to a build-up of placental immunity, which needs to be established independently and in addition to non-pregnant immunity.

On the other end of the risk spectrum lie individuals who possess certain physiological and genetic factors which render them resistant to malarial disease regardless of exposure level. Innate resistance to malaria can result from a range of erythrocyte mutations. Haemoglobinopathies including sickle cell disease and beta thalassaemias are widely recognized as protective against malaria. The sickling of the red blood cells interferes with parasite growth and disrupts the asexual-pathogenic stage of the disease. There is a notable overlap between malaria prevalence and levels of the sickle cell allele, which is manifest in the fact that its frequency exceeds the 10% mark in endemic regions of Africa and reaches 25% in certain places such as Ghana (Aidoo *et al.*, 2002). Another example is the high prevalence of glycophorin B-negative erythrocytes in pygmies. Again, this mutation interferes with parasite growth and keeps parasite densities low (reviewed in Weatherall 1997).

1.5 Innate Immune Participants in *P. falciparum* Infection

Whilst acquired immune responses eventually confer significant protection against malarial pathology, the first-line defense against malaria is likely to come from innate immune cells; e.g. phagocytic granulocytes, macrophages, natural killer cells and possibly $\gamma\delta$ T cells. The profile of cytokines released in the first few hours of malaria infection, as has been demonstrated in humans and mice, may determine the course of disease and the final outcome.

Macrophages

Campos *et al* (2001) showed that macrophages are able to recognize glycosyl-phosphatidylinositol (GPI) anchors on the surface of *Trypanosoma cruzi* through Toll-like Receptor 2 (TLR2) (Campos *et al.*, 2001). The authors extended this observation to several other parasitic protozoa, and even though they did not specifically address *Plasmodium*, there is a high probability that this recognition holds true for this family of protozoans as well, considering the abundance of GPI on the parasite's surface and the high conservation of TLR recognition patterns. The ligation of protozoan GPI to TLR2 resulted in the induction of TNF- α , IL-12 and NO production by the macrophage, which in turn have been shown to upregulate IFN- γ release and to further augment TNF- α release (discussed in more detail in the next two sections). These cytokines can serve as mediators between the innate and the ensuing adaptive response but can also enhance macrophage (Rockett *et al.*, 1991) and neutrophil (Kumaratilake *et al.*, 1991) phagocytic activity. Macrophage phagocytosis has also been shown to be dependent on the developmental stage of *P. falciparum*, with ring-stage parasites being taken up readily and trophozoites being phagocytosed less efficiently (Schwarzer *et al.*, 1992). Another factor that affects the level of antigen uptake by the macrophage is antigen dose, since very high concentrations of parasitized erythrocytes seem to reduce their phagocytic activity (Leitner *et al.*, 1997). As macrophages become increasingly full of indigestible haemozoin (the non-toxic storage form of haemoglobin haem) their phagocytic and antigen presenting abilities seem to become impaired and their cytokine production favors TNF- α release, a factor which may contribute to malaria pathology (Arese *et al.*, 1997)

Dendritic cells

In addition to macrophages, dendritic cells (DC) also serve as part of the front line defense and as a significant source of IL-12 in many infections (Ferbas *et al.*, 1994) (Macatonia *et al.*, 1995). However, their role in malaria infection has not been adequately characterized. There is evidence that *P. falciparum* parasitized erythrocytes bind immature DC through an interaction between PfEMP-1 and CD36, resulting in inhibition of DC maturation, abrogation in IL-12 production, a switch to IL-10 production, and reduction of the subsequent T cell proliferative response (Urban *et al.*, 1999) (Urban *et al.*, 2001). The authors hypothesize that this interaction and downregulation of IL-12 represents a mechanism of parasitic immune evasion, but this seems questionable since other cells present at higher frequencies (at least in peripheral blood) are able to produce IL-12 (Scragg *et al.*, 1999) (Hensmann *et al.*, 2001). Recent data, based on the flow cytometric size and granularity scatter analysis of IL-12 producing cells following *in vitro* stimulation with *P. falciparum*-infected erythrocytes, indicates that macrophages rather than DC are the main source of IL-12 at 18 hours post exposure to parasitized RBC (Walther and Riley, unpublished). However, this finding does not preclude DC-derived IL-12 at earlier time-points, particularly since early DC activation has been shown in the mouse. Using murine DC, derived from bone marrow cultures, and stimulated with *P. chabaudi* *in vitro*, Seixas *et al.* were able to induce IL-12 production from DC within 30 minutes of exposure (Seixas *et al.*, 2001).

NK cells

The importance of IL-12 has been demonstrated in its ability to stimulate natural killer (NK) cells to produce IFN- γ , a cytokine central to both the innate and adaptive response to malaria, as discussed in great detail in the next few sections. NK cells derive from lymphoid progenitor cells originating from the bone marrow and can provide immune protection by directly binding and killing the pathogen (Blanchard *et al.*, 1992), by lysing the infected cell (Oddo *et al.*, 1998) or by secreting IFN- γ and thereby stimulating other cell types (Bancroft 1993). NK cell recognition of target cells is largely dependent on the latter's expression of MHC Class I on its surface. Cells which express normal levels of MHC Class I are spared, whereas cells that do not express it at normal

levels, suggesting an intracellular infection or neoplastic defect, signal the NK cell to kill through cytotoxic mechanisms (Boyington *et al.*, 2000).

In malaria, NK cells have been better characterized in the mouse model, where most studies have underscored their importance in early control of infection. In *P. chabaudi* resistant C57/BL6 mice, depletion of NK cells through administration of anti-NK1.1 monoclonal antibody followed by a challenge infection resulted in a more severe disease course and higher mortality (Kitaguchi *et al.*, 1996). Similarly, using susceptible A/J mice the same protocol resulted in higher parasitemia and 100% mortality, suggesting that early NK activity, most likely in the form of IFN- γ secretion, is central to immunity (Mohan *et al.*, 1997). These results are further supported by experiments done in C57BL/6 IL-12p40 deficient transgenic mice wherein *P. chabaudi* AS blood stage primary parasitemia was significantly increased and infection was not effectively controlled as compared to the wild type. This effect was directly linked to impaired NK stimulation and IFN- γ production (Su *et al.*, 2002). However, another study showed that depletion of NK1.1+ cells in infection with an attenuated variant of *P. berghei* had no effect on parasitemia or IFN- γ production (Yoneto *et al.*, 1999). These contradictory results may be due to the different parasite species or parasite-mouse strain combination used. Overall, it seems that early NK activation and IFN- γ secretion in mice is central to optimal control of infection and recovery from malaria.

NKT cells

There is also a population of cells which shares NK and T cell characteristics, in that both CD56 (NK1.1 in mice) and CD3 are expressed on the cell surface. These cells have also been better characterized in mice than in humans and are aptly named NKT cells. The T-cell receptor of the murine NKT cell pairs the V α 14 chain with the V β 8 chain; the V α 24 and V β 11 chains form the invariant T cell receptor (TCR) of humans NKT cells (Fearon *et al.*, 1996). This invariant pairing differentiates them from CD56+ antigen specific T cells since the latter group of cells uses a more variable repertoire of α and β chains (Slifka *et al.*, 2000). NKT cells have been shown to produce significant levels of IFN- γ very early in infection followed by a rapid switch to IL-4 production, and they are thereby implicated in immune regulation (Matsuda 2000). They recognize target cells

through their non-classical MHC Class I-like CD1d receptor whose natural ligand has yet to be identified (Fearon *et al.*, 1996) (Bendelac *et al.*, 1997). In *P. berghei* ANKA infection, a model for human cerebral malaria, depletion of NK1.1+ T cells either through antiNK1.1 MAb administration (Yanez *et al.*, 1996) or by use of a CD1 deficient mouse strain (Romero *et al.*, 2001) resulted in no differences in the course of infection as compared to the controls. These data suggest that NKT cells are not directly involved in immunity and likewise do not play a role in precipitating infection. However, in *P. yoelii* infection CD1d ^{-/-} mice experienced a prolonged parasitemia. Although these mice ultimately recovered, the authors concluded that this subset of cells is associated with resistance (Mannoor *et al.*, 2001). Nonetheless, NKT cells may prove to be central in vaccine design. As previously stated, the natural ligand for CD1d has yet to be identified. However, the glycolipid alpha-galactosylceramide (α -GalCer), derived from marine sponges but not present in mammals, can activate both murine and human NKT cells through CD1d. It has recently been shown that by co-administering this ligand with suboptimal doses of sporozoites to mice as an adjuvant, their level of protective anti-malarial immunity is increased. This suggests that activation of endogenous CD1d-restricted NKT cells may be involved in the bystander activation of other immune cells thereby mediating the observed enhanced resistance (Gonzalez-Aseguinolaza *et al.*, 2002).

$\gamma\delta$ cells

One of the cell types thought to bridge the innate and the adaptive immune response is the $\gamma\delta$ T cell. It is unclear exactly what role $\gamma\delta$ T cells play in malarial immunity, and to which branch of the immune system they are most closely related. In humans, $\gamma\delta$ cells have been shown to have cytolytic properties (Koizumi *et al.*, 1991) and are able to hinder parasite replication in vitro (Elloso *et al.*, 1994). Elloso *et al.* studied human $\gamma\delta$ cells in vitro and determined that the intraerythrocytic stage of the parasite is not affected by the presence of $\gamma\delta$ cells, and that for cytolytic activity to occur, there needs to be direct contact between the cell and the newly ruptured merozoite. They also found that the expanded V δ 2 subset sustained its level following parasite clearance, whereas the V δ 1 subset declined substantially. They suggested that the underlying

reason for this phenomenon may have something to do with the two subsets recognizing different components of the malarial parasite (Elloso *et al.*, 1994). Along similar lines of preferential antigen recognition, Waterfall *et al.* reported a preferential activation of $\gamma\delta$ cells to live *P. falciparum* antigen as opposed to crude freeze-thawed schizonts when incubated together in vitro for 6 days (Waterfall *et al.*, 1998). The hypothesis is that $\gamma\delta$ cells require a labile parasite product in order to be activated, which is no longer present when the antigen has been killed. These cells have also been implicated in cytokine production. Certain studies have yielded results that strongly support their ability to produce large amounts of inflammatory cytokines, namely IFN- γ , in response to infection (Goodier *et al.*, 1995), whereas others question their importance and demonstrate that levels of IFN- γ remain elevated even when $\gamma\delta$ cells are not activated (Dick *et al.*, 1996). The potential importance of $\gamma\delta$ T cells has also been demonstrated in the mouse, where studies in $\gamma\delta$ T cell-deficient transgenic mice challenged with *P. chabaudi*, showed that the level of parasite clearance was lower as compared to the wild type. In the wild type, the authors identified the responding $\gamma\delta$ T cells as IFN- γ producers, suggesting that effective control of parasitemia may pivot on the ability of these cells to mount an inflammatory cytokine response (Seixas *et al.*, 1999).

1.6 Adaptive Immune Participants to *P. falciparum* Infection

The adaptive immune response primarily depends on the actions of the $\alpha\beta$ T cells and the B cells. About 65% of $\alpha\beta$ T-cells belong to the CD4⁺ subset while the remainder express the CD8⁺ surface marker.

CD8 T cells

Since red blood cells do not express HLA-Class I, the role of CD8⁺ T cells in the erythrocytic stage of infection has not been extensively characterized. However, activation of CD8⁺ T cells by MHC Class I and infected hepatocytes has been implicated in the inhibition of parasite expansion (Aidoo *et al.*, 2000). The cytotoxicity of CD8⁺ T cells is manifested through perforin-mediated and FAS-mediated pathways but this does not seem to play an important role in the development of pathology nor does it seem to be

their primary role during infection. CD8+ T cells are also capable of producing IFN- γ thereby working to kill parasitized cells indirectly (Aidoo *et al.*, 2000), and their ability to produce inflammatory cytokines could also play a role in cerebral malaria by inducing the production of TNF- α and NO derivatives in the cerebral tissues. It has been hypothesized that CD8+ T cells are stimulated to produce IFN- γ by phagocytic cells expressing malaria antigens through MHC class I (Waki *et al.*, 1992) however, this has not been clearly demonstrated. Their function as a source of IFN- γ seems to be quite significant as illustrated in the mouse model. A study in C57BL/6 FAS and perforin knockout mice demonstrated that when immunized with *P. berghei* ANKA sporozoites (liver stage) and later rechallenged, these mice were as protected against disease as the wild-types (Renggli *et al.*, 1997). This suggests that CD8+ T cell cytotoxicity is not a significant mechanism of immunity to malaria. Another study, also using C57BL/6 mice infected with *P. berghei* ANKA, showed a significant abrogation in the development of cerebral malaria following CD8+ T cell depletion- and consequently IFN- γ reduction (Yanez *et al.*, 1996), further strengthening the evidence for a role for CD8+ T cells as cytokine producers. However, the opposite has also been demonstrated by Grau *et al.* who found CD8+ T cell presence or absence had no effect on subsequent disease (Grau *et al.*, 1986) pathology, although they did use a different mouse strain which could account for the contradictory results.

CD4 T cells

Numerous studies have been conducted in experimental models, which support the necessity of CD4+ T cells for controlling malarial infection (Meding *et al.*, 1991) (Podoba *et al.*, 1991). CD4+ T cells are divided into two subgroups, namely Th1 and Th2. The Th1 group serves an inflammatory purpose by producing IL-2, lymphotoxin and IFN- γ , whereas the Th2 helper group produces IL-4, which favors the humoral response and stimulates B-cells and antibody production (Waki *et al.*, 1992). During acute *Plasmodium* infection, CD4+ T cells are thought to migrate to tissues, resulting in a higher number of CD8 T-cells circulating in the peripheral blood (Waki *et al.*, 1992). The selective proliferation of CD4+ T cells to crude schizont extract has been demonstrated by a number of studies (Waterfall *et al.*, 1998), also showing that CD4+ T

cell stimulation with live parasites in vitro is less effective by comparison. CD4⁺ T cells have also been shown to be a significant source of IFN- γ during parasitic infection, also producing IL-2, thereby making them necessary for the proper expansion of the $\gamma\delta$ population (Koizumi *et al.*, 1991). They also seem to be necessary in the development of a strong humoral response, since research shows that CD4⁺ T cell depletion results in an impaired ability to produce antibodies and to clear infection effectively. Studies in SCID mice show that transfer of CD4⁺ T cells alone was insufficient to confer resistance, whereas transferring them in conjunction with B cells rendered the mice able to clear their infection (Meding *et al.*, 1991).

The importance of CD4 cells in malaria is further supported by results from studies looking at *P. falciparum* co-infection with HIV. During HIV infection the virus progressively depletes CD4⁺ T-cells. Whitworth *et al.* (Whitworth *et al.*, 2000) studied the effect of co-infection in a group of HIV-1 infected individuals in rural Uganda and found a correlation between HIV infection and clinical malaria as well as parasitemia, the severity of which increased with progressive HIV disease.

1.7 Acquired immune Responses to *P. falciparum*

Malarial immunity seems to involve the development of both anti-disease immunity as well as anti-parasitic immunity. The evasion of pathology is believed to be associated with neutralization of parasitic toxins and consequently with averting the production of endogenous pyrogens, whereas anti-parasitic mechanisms are responsible for clearing the infection from the circulation (reviewed by Riley 1999).

Anti-parasite immunity

Anti-parasite immunity seems to develop simultaneously with clinical immunity, and is dependent upon both cellular and humoral branches of the immune system. Malarial attacks characteristically decrease in severity as the individual ages; this is accompanied by lower parasite density. This trend seems to indicate that immunity becomes progressively more efficient with every subsequent infection. For example, in order to attain effective anti-parasite immunity, one may need to develop a repertoire of

antibodies capable of blocking parasite invasion and tissue adhesion which are mediated by highly variable or polymorphic proteins (Molineaux 1996). The breadth of this repertoire necessarily expands due to the parasite's ability to modify the antigens expressed on the surface of the erythrocyte once it has invaded. An example is PfEMP1, an antigen involved in parasite sequestration, encoded by a family of *var* genes which undergo frequent non-homologous recombination leading to heterologous expression of PfEMP1 variants by different parasites. Infection with a parasite variant that is not recognized by the existing antibody repertoire may lead to uncontrolled parasite replication and, therefore, pathology. Since clinical immunity develops progressively, a gradual and parallel development of a diverse antibody repertoire could explain the fact that immunity to malaria improves over time (reviewed in Bull *et al.*, 2002). The importance of malaria-specific antibodies to host protection is exemplified by the range of anti-parasitic immune effector mechanisms they provide including inhibition of cytoadherence (Newbold *et al.*, 1992), inhibition of erythrocyte invasion (Guevara Patino *et al.*, 1997), and mediation of antibody dependent cytotoxicity and cellular inhibition (Oeuvray *et al.*, 1994).

Anti-disease immunity

As summarized above, immune effector mechanisms, T-cells and cells of the innate immune response mediate anti-parasite immunity through the regulated production of inflammatory cytokines, namely IFN- γ , TNF- α , IL-12 and IL-18 among others. These cytokines can originate from a number of immune cells, including CD4-Th1 cells, CD8 cytotoxic cells, NK cells and $\gamma\delta$ cells, as well as dendritic cells and macrophages in the case of IL-12 and IL-18. Attaining a state of clinical immunity seems to involve developing the ability to control inflammatory cytokines, and to mediate their release in a highly regulated manner. This hypothesis is supported by the relatively consistent cytokine profiles of individuals suffering clinical pathology versus those able to support infection while averting symptoms. Severe malarial anemia has been linked to high TNF- α levels and low IL-10 levels (Kurtzhals *et al.*, 1998) (Othoro *et al.*, 1999). Histopathology performed on brain tissue of fatal cerebral malaria cases detected a localized buildup of TNF- α , IFN- γ and IL-1 β (Udomsangpetch *et al.*, 1997). However,

resistance to disease is also characterized by the presence of these cytokines, albeit at lower levels. PBMC from clinically immune Gambian donors produced an ample amount of IFN- γ when stimulated with soluble malaria antigens (Riley *et al.*, 1988). Using a similar experimental protocol, Luty *et al* (Luty *et al.*, 1999) showed that PBMC taken from patients presenting with mild disease were more efficient producers of IFN- γ - as compared to those from severe cases- when stimulated with sporozoite or merozoite antigen peptides, thereby associating the production of this cytokine with better control of symptoms. A study conducted in Vietnamese adults with severe clinical disease associated acute organ failure with elevated plasma concentration of inflammatory cytokines and also found that patients who eventually died produced deficient amounts of IL-10 as compared with those who survived (Day *et al.*, 1999). The association between resistance to clinical symptoms and presence of anti-inflammatory mediators has also been documented in the case of TGF- β , where high ratios of pro-inflammatory cytokines to anti-inflammatory ones was associated with increased risk of fever, thereby underlining the importance of a balance between the two (Dodoo *et al.*, 2002). Drawing from the data described, it seems that the same cytokines may be involved in both protection and pathology depending on their absolute and relative concentrations. It is precisely this balance that needs to be characterized in order to be able to abrogate the detrimental pathology of malaria whilst optimizing anti-parasitic effector mechanisms.

1.8 Inflammatory mediators of pathology

When released in controlled amounts and a timely manner, inflammatory mediators can be vital in combating disease. For example, in humans, TNF- α induces innate cells, namely neutrophils and macrophages, to phagocytose parasites (Kwiatkowski 1992). Similarly, in the mouse model, early presence of TNF- α mRNA in spleen cells of C56BL/6 and A/J mice is correlated with resistance to *P. chabaudi* AS infection, whereas its later presence in the serum is associated with mortality (Jacobs *et al.*, 1996a). This correlation holds true for other mouse models as well as for humans, with clinical malaria being associated with the presence of circulating pyrogens and survival depending on the host's ability to contain their systemic production.

TNF- α plays a central role in symptomatology, its elevated production having been repeatedly linked to parasitemia as well as to disease progression (Grau *et al.*, 1989b). TNF- α upregulates mediators that facilitate parasite sequestration, such as the expression of ICAM-1 on the cerebral endothelium to which parasitized red blood cells adhere and thereby damage cerebral blood vessels leading to hemorrhages and coma. TNF- α can also augment the release of NO and oxygen radicals which are known to have antiparasitic effects, but which can also interfere with neurotransmission. One role of TNF- α in its involvement in malaria pathology seems to lie in its ability to upregulate other inflammatory mediators, an important one being IFN- γ (reviewed in Richards 1997).

Although many cytokines have been implicated in malaria protection and pathology, research has exposed IFN- γ as being a pivotal component in the immune response to this pathogen. IFN- γ is produced by T cells ($\alpha\beta$ and $\gamma\delta$) and NK cells (Richards 1997). IFN- γ has the ability to regulate the amount of TNF- α made by macrophages/monocytes, the amount of IL-12 and the amount of IFN- γ itself, all of which act cyclically and stimulate each other's release. IFN- γ can also work to upregulate the expression of both MHC Class I and MHC Class II thereby facilitating the presentation of antigen and driving the adaptive immune response (Lammas *et al.*, 2000). The following sections focus on the specific characteristics of IFN- γ and summarize what has been established concerning its role in malaria infection.

1.9 IFN- γ

IFN- γ belongs to a family of cytokines characterized by their antiviral activity. Interferons α and β are known as type I interferons, and resemble each other, whereas IFN- γ is a type II interferon and has been assigned an immunoregulatory function with its antiviral capabilities being of secondary importance. It was first identified in 1965 (Wheelock, 1965) and the genes encoding it and its receptor were cloned during the 1980s (Gray *et al.*, 1982) (Aguet *et al.*, 1988).

Structurally, IFN- γ is a non-covalently linked homodimeric molecule of 50 kDa, consisting of two identical polypeptide chains. It was originally described as macrophage activating factor (MAF), but IFN- γ is also able to upregulate the expression of MHC class I and has the more unique capability of inducing MHC Class II expression on leukocytes and epithelial cells. It is also able to regulate the functions of mononuclear phagocytes and the release of monokines, the most significant being IL-12 and TNF- α . Additionally, IFN- γ exerts pressure on the humoral immune response by causing antibody IgG heavy chain class switching. Finally, it is also able to regulate itself by controlling the level of IFN- γ receptor-expression on surrounding cells and consequently affecting their responsiveness (reviewed in Farrar *et al.*, 1993).

The IFN- γ receptor was identified on several different cell types in the early 1980s by radioactive-ligand binding studies (reviewed in Farrar *et al.*, 1993). The active form of the receptor includes two subunits. The ligand binding subunit, also known as the IFN- γ receptor α chain or IFNGR1, is involved in binding ligand and is constitutively expressed on the majority of cell types. The second subunit is called the IFN- γ receptor β chain or IFNGR2 and serves as an accessory molecule. It is not as ubiquitous as the α chain, being constitutively expressed on a much lower frequency of cells, but exposure to certain stimuli can markedly upregulate its expression on certain cell types, rendering the given cell receptive to the effects of this cytokine. It has also been demonstrated, in both mice and humans, that cellular exposure to IFN- γ itself can have an effect on downregulating surface β chain expression on target cells, thereby diminishing their sensitivity to this cytokine in a self-regulating loop.

The α and β chains are not closely associated when the cell is in an inactive state. However, once exposed to IFN- γ , two α chains dimerize and form a complex which is recognized by the extracellular domain of the β chain. This association of a pair of α and β chains on either side of the ligand results in the convergence of their intracellular domains, both of which have inactive JAK enzymes attached: JAKs, or Janus family kinases, are protein tyrosine kinases and are the first of two classes of signaling proteins involved in the IFN- γ signaling pathway. Once they are in close proximity, JAK1 and JAK 2, associated with the α and β chains of the receptor respectively, are able to

transactivate each other resulting in the phosphorylation of the Y₄₄₀ residues of the α chains and consequently two docking sites for STAT1 molecules. STATs, or signal transducers and activators of transcription, are the second class of signaling proteins involved in this pathway. They are latent cytosolic transcription factors which exist freely in the cytoplasm until recruited into a signaling cascade. Several STATs have been identified in mammals, however the IFN- γ pathway exclusively involves STAT1, which docks on the α chain and becomes tyrosine phosphorylated before detaching to form homodimers. These complexes are then phosphorylated on a particular serine residue on the C-terminal end and translocate to the nucleus (reviewed by Bach *et al.*, 1997).

Once in the nucleus, the STAT1 homodimers bind to specific DNA regions, namely γ -IFN activating sites, or GAS elements. These GAS elements drive the transcription of neighboring IFN- γ -inducible genes (examples are guanylate binding protein-1, MHC class II transactivator, interferon regulatory factor-1), which are rapidly activated since their transcription is not dependent on the synthesis of new proteins. An additional factor which contributes to the specificity of genes ultimately transcribed following an IFN- γ signal, is the nuclear association of the STAT1 homodimer with different transcriptional factors. For example, Sp1 interacts with STAT1 and induces the transcription of the ICAM-1 gene (reviewed in Stark *et al.*, 1998).

Once IFN- γ is ligated to its receptor and the signaling cascade is underway, the cell internalizes the entire complex into an acidified compartment, facilitating the dissociation of the IFN- γ molecule and its subsequent transfer to and degradation in a lysosome (Bach *et al.*, 1997). IFN- γ molecules are therefore not recycled and re-released. However, IFN- γ gene transcription is rapid and the cytokine is released immediately following its synthesis. Therefore, IFN- γ mRNA is detectable as early as 6 hours following stimulation and the cytokine itself can be measured by about 8 hours, with the production of both mRNA and IFN- γ reaching a maximum level at about 24 hours (Farrar *et al.*, 1993).

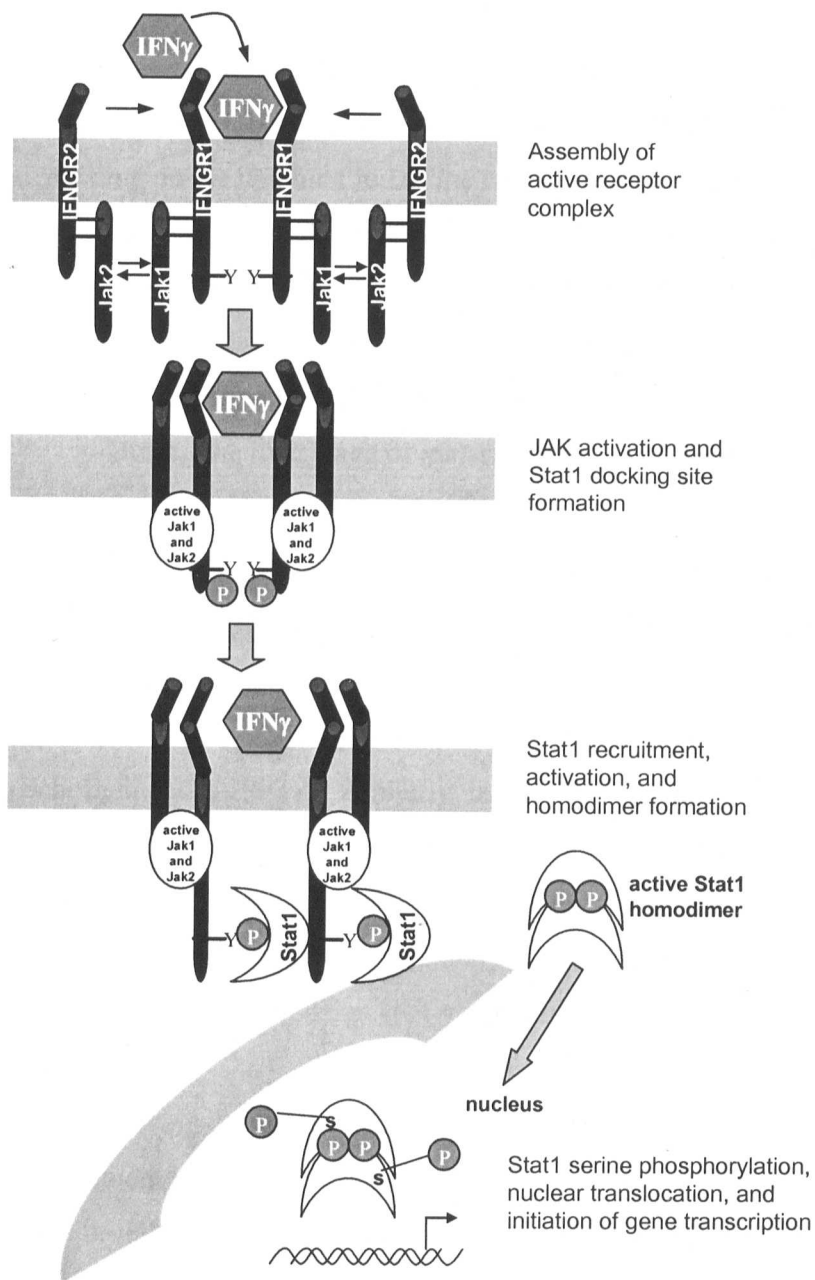


Figure 1.3: IFN- γ signaling pathway. This figure has been taken directly from *Stark et al. Annu. Rev. Biochem.* 1998. 67:227-264 and illustrates the steps involved in IFN- γ signaling. A detailed description of this sequence is included in the text.

1.10 IFN- γ Responses to Malaria Infection

Mouse

In malaria infections, IFN- γ has proved necessary in controlling parasitemia and in ensuring survival, both in mice and in humans. The mouse models for malaria can vary depending on the combination of the mouse strain and the parasite species or strain chosen. Based on which human symptoms, disease course or outcome are being modeled, the pairing will be different. However, the mouse model has revealed an indisputable central role for IFN- γ both in protection and pathogenesis and seems to foreshadow information subsequently gathered from humans.

During the liver stage of malaria infection in mice, it becomes apparent that IFN- γ plays a dual role, the CD4 T-cell production of IFN- γ is associated with protection whereas IFN- γ from CD8 cells seems to be pathogenic (Waki *et al.*, 1992). However, CD8-derived IFN- γ is necessary for the development of immunity (Doolan *et al.*, 2000) and a complete lack of IFN- γ is associated with the reversal of immunity in previously immunized mice, resulting in the subsequent expansion of exo-erythrocytic parasites (Schofield *et al.*, 1987a). Similarly, in the rhesus monkey, treatment of *P. cynomolgi* B sporozoite infection with recombinant human IFN- γ suppressed infection (Maheshwari *et al.*, 1986). It was previously thought that the central role for CD8 T cells in the liver stage of infection was their cytotoxicity against infected hepatocytes (Hoffman *et al.*, 1991); now it is believed that IFN- γ production is their primary function (Doolan *et al.*, 2000).

Moving on to the erythrocytic stages of infection, the same patterns of IFN- γ involvement hold true. *P. yoelii* and *P. berghei* mouse malarias are used to mimic lethal and non-lethal infections in humans. Mice infected with lethal strains of these parasite species and injected with anti-IFN- γ MAb, succumbed to disease faster than infected IFN- γ competent mice, thereby leading to the conclusion that IFN- γ production is associated with survival (Kobayashi *et al.*, 2000). This observation is further supported by data in non-lethally infected mice, where parasite clearance was preceded by a spike in IFN- γ mediated by T and NK cells, a phenomenon not seen in lethally infected mice

(De Souza *et al.*, 1997). It has been suggested that IFN- γ contributes to enhanced survival by playing an anti-parasitic role as seen in *P. berghei* non-lethal infections, where it stimulates phagocytosis of circulating parasites by macrophages (Yoneto *et al.*, 1999).

Interestingly, TNF- α levels increased equally in lethal and non-lethal infections, suggesting that this cytokine is not integrally involved in protection, but rather is upregulated as a consequence of the production of other immune factors (De Souza *et al.*, 1997). This possibility is supported by data from *P. berghei* ANKA infection in mice susceptible to development of cerebral malaria, where early neutralization of IFN- γ (specifically CD4 T cell-derived IFN- γ) resulted in lower TNF- α serum levels as well as lower IL-12 expression in the brain, leading to evasion of cerebral symptoms. Although mice still died of anemia and high parasite load, these data propose a pivotal role for IFN- γ in the microvascular pathology leading to cerebral disease (Grau *et al.*, 1989a). In *P. chabaudi* AS infections in resistant mice, symptoms most closely resemble those of uncomplicated malaria in humans. Experiments using this malaria model have elucidated the necessity for IFN- γ in survival. Eliminating this cytokine resulted in significant elevation of the primary parasitemia, whether IFN- γ was blocked by administration of anti-IFN- γ MAb (Stevenson *et al.*, 1990a; Stevenson *et al.*, 1990b) or by knocking out either the IFN- γ receptor gene (Balmer *et al.*, 2000; Favre *et al.*, 1997) or the IFN- γ gene itself (Su *et al.*, 2000). The only difference in survival was seen with IFN- γ receptor deficient transgenic mice (IFN- γ $-/-$) which displayed a higher mortality (Balmer *et al.*, 2000; Favre *et al.*, 1997) as opposed to the MAb-treated mice which survived (Stevenson *et al.*, 1990a). Furthermore, IFN- γ $-/-$ mice presented with diminished macrophage-derived IL-12 production both in vivo and in vitro as well as decreased NO release, both factors otherwise associated with successful recovery from infection, leading the authors to conclude that IFN- γ is necessary for the development of protective immunity (Su *et al.*, 2000). In support of their view, an additional study showed that raised serum levels of IL-12 in resistant mice triggered a protective pro-inflammatory reaction in response to erythrocytic infection (Sam *et al.*, 1999). Supplementing resistant mice with recombinant IFN- γ further delayed and decreased parasitemia (Meding *et al.*, 1990). What becomes

apparent from the mouse models is that IFN- γ is pivotal to the progression to cerebral malaria, but that it is also absolutely essential for control of parasitemia. Thus, the conclusion that can be drawn is that the timeliness of the IFN- γ response and subsequent shift to a Th2 or anti-inflammatory type T regulatory response is crucial to combating infection.

Human

As in the mouse, in humans IFN- γ has been associated with both beneficial and detrimental effects. Since the human host obviously cannot be manipulated, much of the data collected on the effects of the relative presence or absence of IFN- γ is circumstantial, the only manipulation being done *in vitro*. In a rare study looking at human experimental malaria, whereby volunteers were infected with sporozoites and their immune response followed as infection developed, an increase in serum IFN- γ concentration preceded the onset of fever, indicating that IFN- γ is involved in the regulation of pyrogen production, most probably TNF- α (Harpaz *et al.*, 1992). Also, individuals who are heterozygous for specific polymorphisms in the IFNGR1 gene promoter are protected from cerebral malaria and death. However, it was unclear from this study how this polymorphism translated to variations in the amount and timing of IFN- γ production (Koch *et al.*, 2002). Overall, it seems that the magnitude of the cytokine response and eventual imbalance of pro and anti-inflammatory responses are important determinants of mortality (Day *et al.*, 1999).

Immunity to the liver stage of *P. falciparum* has largely been studied *in vitro*. IFN- γ has been shown to directly target hepatocytes by binding to their surface and subsequently altering the intracellular environment rendering it unable to support parasite growth, thereby inhibiting sporozoite development (Ferreira *et al.*, 1986; Schofield *et al.*, 1987b). The parasitocidal effects of IFN- γ are further observed when it is added to cultured parasites *in vitro*, resulting in a lowered parasitemia, suggesting that the same may take place during a natural infection *in vivo* (Chizzolini *et al.*, 1990).

The beneficial effects of IFN- γ are not only restricted to the liver. In the blood stage, IFN- γ seems to augment neutrophil phagocytic activity resulting in enhanced

clearance of merozoites (Kumaratilake *et al.*, 2000). Also, a study involving symptomatic Gabonese patients drew an association between inability to control parasite replication and ensuing hyperparasitemia with a lower frequency of IFN- γ producing CD4 T cells as compared to individuals who were able to successfully contain the infection (Winkler *et al.*, 1999). Overall, it seems that a strong and timely IFN- γ response as measured in serum levels of acute, uncomplicated malaria cases is indicative of an effective pro-inflammatory strike of the immune system on the parasite (Torre *et al.*, 2002).

However, producing IFN- γ arbitrarily does not necessarily protect from disease progression. The amount and the timing of IFN- γ release are seemingly critical components of the inflammatory response, a slight imbalance in either conceivably resulting in uncontrolled infection and/or fatal pathology. For instance, a persistent pro-inflammatory response was linked to lingering anemia in treated individuals even after infection was cleared (Biemba *et al.*, 1998). Although a baseline level of this cytokine seems to be necessary to clear parasitemia, high systemic levels are correlated with severe pathology. Due to IFN- γ 's ability to activate macrophages to produce endogenous pyrogens (TNF- α , IL-1 and IL-6), its presence in large amounts can be exceedingly detrimental (Pala *et al.*, 2000; Waki *et al.*, 1992). The severity of these symptoms has been directly correlated with corresponding levels of IFN- γ , with high levels being found in symptomatic individuals and much lower levels where clinical pathology is not apparent (Mshana *et al.*, 1991). In addition, IFN- γ release can also be detrimental in certain types of patients, most notably during pregnancy, where a pro-inflammatory response in the placenta resulting from maternal infection can have fatal consequences on the fetus (Fried *et al.*, 1998).

1.11 Aims of this project

The severity of disease in malaria infection has been correlated with levels of IFN- γ and TNF- α production. I hypothesize that clinical immunity is associated with the ability to regulate the production of pro-inflammatory cytokines to an intermediate level, which allows parasite clearance while simultaneously avoiding severe pathology. In order to fully understand these immunological mechanisms and their role in the acquisition of clinical immunity to malaria, the specific pathways of cytokine production need to be revealed.

The purpose of this project was to elucidate the cellular origins of IFN- γ production in response to *P. falciparum* as a function of time and extent of previous exposure to the parasite. Early on in the research, it became evident that the cells of the innate immune system could be rapidly activated to produce inflammatory cytokines in naïve individuals. Further studies, therefore, focused on the activation of innate immune responses by *P. falciparum*. The aims of the project were thus:

- To identify the cellular source of IFN- γ in naïve and malaria-immune individuals over time, following *P. falciparum* exposure
- To determine the innate sources of IFN- γ production during the early (24 hour) response to *P. falciparum*
- To determine whether NK cell IFN- γ production is IL-12 and IL-18 dependent
- To determine whether NK cell:parasite contact occurs and if it is necessary for optimal IFN- γ production by these cells
- To determine if donor heterogeneity in NK-derived IFN- γ production can be explained through differences in NK cell surface receptor expression and genotype

Chapter 2 General Materials and Methods

2.1 Blood Donors

The UK donors were recruited at LSHTM through the anonymous blood-donation system. Donors who had volunteered to give blood for research were approached by the phlebotomist and asked to donate up to 50 ml blood, up to once every three weeks. Donor characteristics were noted by the phlebotomist at the first bleeding and the ones relevant to these studies are summarized in Table 4.1. Ethical approval for the use of their blood in the studies described throughout this thesis was given by the LSHTM Ethics Committee (application #805).

Donors were also recruited through a collaboration with the Hospital for Tropical Diseases (HTD). Ethical approval was granted by the Joint UCL/UCLH Committees on the Ethics of Human Research (application #00/0050). Patients recently recovered from malaria were bled during their convalescence to verify clearance of parasites and for immunological studies including assays of acquired humoral and cellular immunity. These donors are indicated by the HTD prefix followed by a number.

Ghanaian donors were recruited (as part of a larger study) from the village of Dodowa, approximately 10 miles northeast of the capital, Accra. Dodowa is endemic for *P.falciparum* and transmission occurs year-round (Afari *et al.*, 1995). Therefore, adults in this community have almost certainly been repeatedly challenged with the parasite and suffer only the mildest symptoms upon re-infection. Due to the large volume of blood required for PBMC separation and flow cytometry, the donor pool for this study was limited to adults and mature adolescents. Judging from the transmission frequency in Dodowa as well as previous studies conducted in this area (Dodoo *et al.*, 2002), we assume that they are clinically immune. Donor characteristics for this group are summarized in Table 3.1. Ethical approval for this study was given by the LSHTM Ethics Committee (application #589).

2.2 In vitro *Plasmodium falciparum* culture

P.falciparum parasites were cultured in order to obtain parasite antigens for use in the cell culture assays. Parasites of the 3D7 strain were grown in 25 ml³ flasks (SLS, Nottingham, UK) in 10 ml cultures containing 0.25 ml packed red blood cells of blood type A+ (obtained from the National Blood Service, London), and 9.5 ml complete growth medium. Incomplete growth medium was prepared in one liter batches using the following recipe: 10.4 g RPMI powder (Sigma, Dorset, UK), 5.96 g Hepes (Sigma), 50 mg hypoxanthine (Sigma), and 2.33 g bicarbonate powder (Sigma) made up to 1 liter with distilled H₂O. Incomplete medium was divided into 250 ml aliquots and stored at –20°C until needed. Normal human serum (National Blood Service), also stored at –20°C, was added at 5 ml/50 ml. Complete growth medium was made every week, or more often as needed.

The medium in the cultures was changed at approximately the same time daily and a thin blood smear of each flask was made to determine the level of parasitemia. Blood smears were prepared by smearing a drop of parasitized blood on a microscope slide, air drying and then fixing with methanol for a few seconds before staining with freshly prepared 10% Giemsa (Sigma) in Giemsa buffer. Giemsa buffer consisted of 0.001% di-sodium hydrogen orthophosphate anhydrous and 0.0007% potassium dihydrogen orthophosphate made up in dH₂O to a pH of 7.2 . The buffer was stored at room temperature in a foil covered flask to prevent algal growth. Slides were stained for 10 minutes, washed under the tap and subsequently air-dried. A drop of oil was placed on the edge of the smear where cells were most likely to be evenly spaced, and the slide was examined under the microscope at x100.

Using a blunt needle, flasks were gassed for approximately 30 seconds with 1% Oxygen, 3% Carbon dioxide and 96% Nitrogen. Lids were tightened and flasks were stored at 37°C lying flat on their sides to allow for maximal gas exchange. Once the parasitemia reached a level above 6-7%, the parasites were subcultured and split into two flasks. Each new culture was supplemented with a few drops of fresh uninfected RBC. Prior to being added to the culture, these fresh RBC cells were washed twice in incomplete medium to remove clotting factors.

2.3 Preparation of schizont extract

The 3D7 parasite cultures were on a 42-44 hour cycle. At the correct developmental time-point and parasitemias of 6-7%, schizonts were harvested to prepare dead parasite antigen. First the cultures were centrifuged at 2000 rpm for 5 minutes and any excess medium was removed. Cells were then resuspended at 50% hematocrit and 2 ml aliquots were carefully layered onto 5 ml of density gradient made with 60% Percoll (Sigma) 34% RPMI (Invitrogen, Paisley, UK) and 6% 10x phosphate buffered saline (PBS). The cells were then spun at 2000 rpm for 10 minutes with the brake off. Following the spin, trophozoite and ring stage parasites went through to the bottom of the tube whereas the schizonts formed a distinct reddish-brown band at the top of the Percoll. This layer was then carefully removed and washed twice by resuspending it in incomplete medium and spinning at 2000 rpm for 10 minutes after each wash. The schizont pellet was finally resuspended in 1 ml complete medium and a 5 µl aliquot was transferred to a Neubauer hemocytometer to determine the schizont concentration. The concentration was adjusted to 3×10^7 infected RBC/ml, and the schizonts were placed in an eppendorf tube and freeze thawed three times (liquid nitrogen to 37°C water bath) to lyse the red blood cells and release the contents. *P. falciparum* schizont lysate (PfSL) was stored at -70°C until used.

2.4 Preparation of live parasite antigen

Live parasite antigen was prepared in much the same way as the schizont extract. At time points where the parasites were in the late trophozoite or schizont stage, the separation procedure on Percoll was used as above. Parasites were washed and counted in the same way and placed directly into the cell culture assay wells at the correct pre-determined concentration.

2.5 Mycoplasma detection by PCR

Mycoplasmas consist of a family of microorganisms which frequently contaminate cell cultures. Many mycoplasma species are capable of activating macrophages to release pro-inflammatory mediators which can lead to erroneous results in certain experimental

protocols (Muhlradt *et al.*, 1997). Malaria culture preparations have been shown to be susceptible to mycoplasma contamination, often becoming reinfected even after treatment (Rowe *et al.*, 1998). Since the data included in the following chapters rest on the identification of *P. falciparum*-specific pro-inflammatory cytokine induction from lymphocytes, it was important to ensure parasite preparations and cultures were mycoplasma-free at all times. The contamination status of the cultures was continually monitored using a PCR Mycoplasma Detection Kit (Takara Shuzo Co. Ltd) to test culture supernatants each time a schizont preparation was made, using a nested PCR on the 16S - spacer-23S region of the rRNA operon. The length and sequence varies between mycoplasma species.

The initial reaction of the nested PCR comprised a total volume of 100 µl (69.5 µl H₂O, 10 µl 10x PCR Buffer, 8 µl dNTP Mixture, 1 µl MCGp F1 Primer, 1 µl MCGp R1 Primer, 0.5 µl *TaKaRa Taq* and 10 µl of the sample). The first reaction involved an initial 30 second denaturation step carried out at 94°C followed by 35 cycles of amplification (30 seconds at 94°C denaturation, 2 minutes at 55°C annealing, 1 minute at 72°C extension). The subsequent reaction of the nested PCR also comprised a total volume of 100 µl (78.5 µl H₂O, 10 µl 10x PCR Buffer, 8 µl dNTP Mixture, 1 µl MCGp F2 Primer, 1 µl MCGp R2 Primer, 0.5 µl *TaKaRa Taq* and 1 µl of 1st PCR product) as well as an initial 30 second denaturation step at 94°C followed by 30 cycles as described for the first reaction.

Analysis of the amplified products was done by agarose gel electrophoresis to verify the product and its size. The expected PCR product ranged from 145 to 237 base pairs depending on the presence of any of 13 Mycoplasma species the PCR kit was designed to detect. No positive cultures were ever found, but had a PCR product been detected, the cultures would have been discarded and an aliquot of a fresh, uncontaminated culture would have been obtained for future experiments.

2.6 Peripheral Blood Mononuclear Cell (PBMC) Extraction

Up to 50 ml of venous blood was collected into sterile tubes with heparin at a concentration of 10 iu/ml blood (CP Pharmaceuticals, Wrexham, UK). The blood was

diluted 1:1 in RPMI 1640 and layered either in 10 ml aliquots onto 4 ml of Histopaque (Sigma) in 15 ml centrifuge tubes or in 25 ml aliquots onto 15 ml Histopaque in 50 ml centrifuge tubes. The cell suspension was then spun at 1350 rpm for 30 minutes with the brake off. The PBMC formed a distinct white band above the Histopaque; this band was removed and washed twice in RPMI, spinning at 2000 rpm for 10 minutes after each subsequent wash. The cell pellet was finally resuspended in complete cell growth medium (RPMI 1640, 5% autologous serum, 100 Units/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine (Invitrogen)) and a small aliquot was diluted 1:10 in Trypan blue to count the cell concentration.

2.7 PBMC Culture

After counting, cells were resuspended to a concentration of 10^6 cells/ml in growth medium (RPMI 1640, 5% autologous serum, 100 Units/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine (Invitrogen)) and one million cells were placed in each well of a Nunc 24-well flat bottom tissue culture plate (SLS Ltd, Nottingham, UK) together with the appropriate antigen, mitogen or control stimulant. The cultures were then incubated at 37°C in an atmosphere of 5% carbon dioxide for the required amount of time.

2.8 Cell Surface and Intracellular Cytokine Staining for Flow Cytometry

The Golgi transport blocker Brefeldin-A (Sigma) was added at 10 µg/ml to cells for the last 3 hours of the incubation, in order to prevent the release of intracellular cytokines. After the appropriate incubation period, 400 µl of supernatant from each well were collected and stored at -70°C for analysis by ELISA. Then, cells were transferred to centrifuge tubes and wells were washed with 2 ml of FACS buffer (1x PBS, 0.1% NaN₃, 1% FCS) in order to collect any additional cells. Cells were then spun at 2000 rpm for 10 minutes and the pellet was resuspended in FACS buffer for a second wash. The cell pellets were then resuspended in FACS buffer. The cell suspensions were divided into 100 µl aliquots, each of which was placed in a separate well of a Nunc 96-well

round-bottom microtiter plate (SLS) along with the relevant fluorochrome-labeled monoclonal antibody to stain for cell surface markers. Antibodies were titrated to determine the amount needed, and results of these titrations are displayed in table 2.3. The plates were incubated in the dark at 4°C for 30 minutes followed by two consecutive washes with FACS buffer to eliminate any unbound antibody.

Cells were then resuspended in 100 µl fixation buffer (1x PBS, 4% paraformaldehyde) for 15 minutes at room temperature in the dark and washed once with FACS buffer. In order to stain for intracellular cytokine, the cells were resuspended in 100 µl permeabilization buffer (1x PBS, 1 % saponin, 0.1% sodium azide) to which was added 1 µl of either anti-IFN-γ or anti-TNF-α antibodies, depending on the assay. The plates were incubated again in the dark at 4°C for 30 minutes and followed by another wash with FACS buffer. Finally, the cell suspensions were diluted in 200 µl of FACS buffer and transferred into Falcon flow cytometry tubes in order to be read by the flow cytometer.

Cells were analyzed using Becton Dickinson's FACScan flow cytometer and CELLQuest software. A total of 100,000 events were collected from each sample for analysis, unless otherwise stated.

2.9 Interferon-γ ELISA

The IFN-γ ELISA was carried out over the course of three days. All samples for any particular data set were assayed at the same time. On the first day of the assay, flat-bottomed immulon-4 plates (Thermo Life Sciences, Basingstoke, UK) were coated with an anti-human IFN-γ monoclonal antibody (Pharmingen, 1mg/ml stock concentration) diluted in carbonate coating buffer (0.1M NaHCO₃ pH 8.2) at a final concentration of 2µg/ml. Each well was loaded with 50 µl of the diluted antibody and plates were incubated overnight at 4°C.

On the second day, plates were washed four times in washing solution (48.5g PBS/liter dH₂O + 0.05% Tween-20) and 150 µl of blocking solution (10% heat-inactivated FCS in PBS) was added to each well and incubated at room temperature for two hours. Plates were then washed again twice with washing solution and 100 µl of

Monoclonal antibody	fluorochrome	isotype	manufacturer	amount
CD3	TRI	Mouse IgG-2a	Caltag (Towcester, UK)	1 μ l
CD56	FITC	Mouse IgG ₁	Becton-Dickinson (Oxford, UK)	2 μ l
CD4	FITC	Mouse IgG ₁ , κ	Becton-Dickinson	2 μ l
CD8	TRI	Mouse IgG-2a	Caltag	2 μ l
TCR- $\alpha\beta$	FITC	Mouse IgG ₁ , κ	Becton-Dickinson	2 μ l
TCR- $\gamma\delta$	FITC	Mouse IgG ₁ , κ	Becton-Dickinson	2 μ l
IFN- γ	PE	Mouse IgG ₁ , κ	Pharmingen (Cowley, UK)	1 μ l
TNF- α	PE	Mouse IgG ₁ , κ	Pharmingen	1 μ l
Human Glycophorin A	PE	Mouse IgG ₁ , κ	Caltag	1 μ l
Isotype Control	FITC	Mouse IgG ₁ , κ	Becton-Dickinson	2 μ l
Isotype Control	PE	Mouse IgG ₁ , κ	Pharmingen	1 μ l
Isotype Control	TRI	Mouse IgG-2a	Caltag	1 μ l
Isotype Control	PE	Mouse IgG ₁ , κ	Caltag	1 μ l

Table 2.1: Monoclonal antibodies used for FACS staining. Each fluorescently labeled antibody was titrated to determine the minimum amount needed to saturate the system. Each antibody is summarized in this table in terms of fluorescent label, isotype, and manufacturer. The last column refers to the amount of stock antibody added to 100 μ l of cell suspension containing approximately 500,000 cells.

each sample supernatant was added to duplicate wells. Recombinant standard IFN- γ (Pharmingen) was diluted in standard diluent (RPMI 1640, 5% heat-inactivated human AB serum, filtered through 0.2 μ m filter) and doubling dilutions from 2000 pg/ml to 31 pg/ml were plated in duplicate down the left side of every plate. Again, plates were incubated overnight at 4°C.

On the third day, plates were washed four times and 100 μ l of biotinylated anti-human IFN- γ MAb (Pharmingen, 0.5 mg/ml stock concentration) was added to each well at a concentration of 1 μ g/ml diluted in blocking solution. Plates were incubated at room temperature for 45 minutes and subsequently washed again four times. Each well was then loaded with 100 μ l of Avidin Peroxidase (Sigma) at a concentration of 2.5 μ g/ml, again diluted in FCS-PBS and incubated for 30 minutes at room temperature.

Finally, plates were washed carefully five times and 100 μ l of o-phenylenediamine (OPD) solution in citrate-phosphate buffer (1 part 0.2M Na₂HPO₄: 1 part 0.1M citric acid: 2 parts dH₂O, 0.4 mg/ml OPD, 0.03% H₂O₂) was added to each well. Plates were developed in the dark at room temperature for approximately 15 minutes and stopped with 50 μ l/well of 2M H₂SO₄. Optical densities were read at 492 nm (Dynex Technologies, Chantilly, Virginia, USA) and IFN- γ concentrations were calculated by reference to a standard curve using Revelation software (Dynex Technologies). Values above the top of the standard curve (2000 pg/ml) were retested at 1/10 and 1/100 dilutions in RPMI and cytokine values were recalculated. The lower limit of detection was set at 62 pg/ml based on the lowest point on the linear part of the standard curve, as determined after examining all standard curves.

2.10 Interleukin-12 ELISA

Immulon 4, 96 -well, flat-bottom microtiter plates (Thermo Life Sciences) were coated with 50 μ L /well of mouse anti-human IL-12 monoclonal Ab (R&D Systems, Abingdon, UK) in PBS (pH 7.4) at 4 μ g/ml (stock 500 μ g/ml), and incubated at 37°C for 2 hours. Plates were washed 4 times with washing solution (48.5g PBS/liter dH₂O + 0.05% Tween-20). Plates were subsequently blocked with 300 μ L /well blocking buffer (4% BSA, 5% sucrose in PBS) for 1 hour at RT and then washed twice. Then, 50 μ L

/well of 2-fold serially diluted recombinant IL12p40 standard (8,000pg/ml to 125pg/ml in 0.4% BSA in RPMI 1640) (R&D Systems) and culture supernatant was added to duplicate wells and incubated at RT for 2 hours with gentle shaking.

Following 4 washes, 50 μ L/well of 0.2 μ g/ml biotinylated mouse anti-human cytokine monoclonal Ab (R&D Systems) (diluted in 0.4% BSA, 0.05% Tween 20 in Tris buffered saline- 20mM Trizma base, 150 mM NaCl) was added to each well and the plates were incubated for 2 hours at RT with gentle shaking. Again, plates were washed four times and 50 μ L /well of avidin-labeled horseradish peroxidase (HRP; Sigma) at 1/10,000 of stock solution (diluted in the same solution used for the biotinylated step) was added to each well for 20 minutes at RT. Wells were washed thoroughly (8 times) with washing solution and 100 μ l per well of 0.4 mg/ml OPD substrate in citrate-phosphate buffer were added, as well as 0.4 μ l/ml H₂O₂ immediately prior to use. Plates were developed in the dark for 20-30 minutes and stopped with 50 μ L/well of 2M H₂SO₄. Optical densities were read at 492 nm (Dynex Technologies) and IL-12 concentrations were calculated by reference to a standard curve using Revelation software (Dynex Technologies). The lower limit of detection was set at 125 pg/ml based on the lowest point on the linear part of the standard curve, as determined after examining all standard curves.

2.11 NK cell lines

NK cell lines and clones were prepared with the assistance of Konstantina Eleme as part of a collaboration with the laboratory of Dr. Daniel Davis at Imperial College, London. Heparinized blood from LSHTM donors was transported to Imperial College where PBMC were immediately separated according to the protocol described in section 2.6. Thirty million cell aliquots were resuspended in 10 ml of RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) (Sigma), 2 mM L-glutamine, 1x non-essential amino acids, 1 mM sodium pyruvate, 50 units/ml penicillin-streptomycin and 50 μ M 2-mercaptoethanol (all from Invitrogen). Each 10 ml cell suspension was plated in a Nunc 10 cm tissue culture plate (SLS Ltd) and incubated at 37°C/5% CO₂ for 3 hours to remove monocytes. Plate supernatants were centrifuged for 10 minutes at 270 x g, resuspended at a higher concentration, counted, and subsequently adjusted to 10⁶

cells/ml. Primary Blood Lymphocytes (PBL) were cultured in a 4:3 ratio with irradiated (20,000 rads) 721.221 cells in 2 ml RPMI 1640 supplemented with 10% human AB⁻ serum (Sigma), 2 mM L-glutamine, 1x non-essential amino acids, 1 mM sodium pyruvate, 50 units/ml penicillin-streptomycin and 50 μ M 2-mercaptoethanol (RPMI plus human serum).

Following a week's incubation, PBL were stained with CD3-FITC monoclonal antibody (Becton Dickinson) and CD3-negative cells were sorted in a Beckman Coulter cell sorter. Sorted cells were resuspended in RPMI plus human serum supplemented with 100 U/ml hrIL-2 (Roche, East Sussex, UK) at 10^6 cells/ml/well and mixed with irradiated 721.221 cells (20 minutes, 9830 rads) in a 4:3 ratio (10^6 PBL: 7.5×10^5 irradiated 721.221). Cells were supplemented with fresh medium containing IL-2 every 4-5 days (until they showed impaired cytotoxicity against susceptible targets) and screened periodically by flow cytometry to verify their CD3-negative status.

2.12 NK cell clones

PBMC were separated from buffy coat residues (National Blood Transfusion Service, London, UK) according to the protocol outlined in section 2.6 and irradiated (6,000 rads). PBMC were then mixed with irradiated RPMI8866 cells (12,000 rads) in a 20:1 ratio in DME medium supplemented with 10% AB- human serum, 30% Nutrient Mixture F-12 (Hams), 2 mM L-glutamine, 1x non-essential amino acids, 1 mM sodium pyruvate, 50 units/ml penicillin-streptomycin, 50 μ M 2-mercaptoethanol (all from Invitrogen), 100 units/ml hrIL-2 (Roche) (collectively referred to as DMEM plus supplements) and 1 μ g/ml PHA (Sigma). PBMC and RPMI8866 cell mixtures were plated in U-bottom 96 well plates at 10^5 and 5×10^3 cells per well, respectively, and cultured overnight at 37°C/5% CO₂. Subsequently, PBL from the donor of interest were separated as previously described, stained for surface CD3, and one CD3⁻ cell was sorted into each well containing the irradiated cells and supplemented with 100 μ l of DMEM plus supplements. Expanded cells were split every 4-5 days in a 1:1 ratio in DMEM plus supplements and periodically monitored to verify their CD3⁻ status. Since IL-2 activates

NK cells, in order to avoid erroneous results, all NK cell line and clone experiments were undertaken minimum of 4 days post hrIL-2 restimulation.

2.13 DNA Extraction

Following PBMC separation, the pellet containing RBC and granulocytes was washed twice in RPMI 1640 and then stored at -20°C until needed as a source of DNA. Pellets were thawed and incubated in 0.1% saponin-PBS for 10 minutes at room temperature to lyse the red blood cells, before being centrifuged at 2000 g for 15 minutes at 4°C. The cell pellets were then washed 3 times in PBS and spun at 200 g for 5 minutes at 4°C. Following the washes, pellets were resuspended in lysis buffer (100mM NaCl, 10 mM Tris.Cl pH8.0, 25mM EDTA, 0.5% SDS and 1.4 mg/ml proteinase K made up in dH₂O) and incubated overnight at 37°C. The next day, they were transferred to eppendorf tubes and mixed with an equal volume of phenol, being left to stand at room temperature for 5 minutes before being spun at 5000 g for another 5 minutes at 20°C. The upper aqueous layer was taken off and mixed with an equal volume of phenol/chloroform/iosamyl 25:24:1, left to stand for 5 minutes and centrifuged as before. The upper layer was again removed, added to two volumes of ice cold 100% ethanol and incubated at -70°C for one hour. Tubes were centrifuged at top speed for 15 minutes at 4°C and the supernatants were discarded. The pellets were resuspended in 1 ml of 70% ethanol and centrifuged as before. DNA pellets were then air dried and sent at room temperature to Peter Parham's laboratory at Stanford University for analysis of KIR gene polymorphisms. Ethical approval for this research was granted by the Stanford University Administrative Panel on Human Subjects in Medical Research (application #M1272-04).

2.14 Statistical Analysis

All statistical analysis was performed using Stata 7 Software (Stata Corporation, College Station, Texas, USA). In instances where data were normally distributed, an unpaired Student's t-test was used, with the exception of cases where values were being compared for the same donor in which case a paired t-test was used instead. When data was not normally distributed or sample size was very small, the non-parametric Wilcoxon

signed rank test (for paired data) or the Mann-Whitney test (for unpaired data) were used to assess statistical significance instead.

Chapter 3 IFN- γ Induction and Immunity

3.1 Introduction

As described in Chapter 1, IFN- γ is a key cytokine in malaria infection functioning as both a protective and a detrimental mediator in the course of disease. IFN- γ can synergize with TNF- α to optimize the release of nitric oxide which, in turn, is associated with parasite killing (Jacobs *et al.*, 1996b), (Rockett *et al.*, 1991). Mice deficient in the IFN- γ receptor and infected with *P. chabaudi* were unable to resolve primary infection (Favre *et al.*, 1997). However, in the *P. vinckei* model, neutralizing IFN- γ resulted in the abrogation of mortality (Kremsner *et al.*, 1992). These two studies illustrate the opposite extremes of the effects that IFN- γ can have in malaria infection.

These differences might simply be due to the use of different mouse model systems, but another explanation might hinge on the timing, site or amount of the IFN- γ release. For example, a non-lethal infection correlates with the mouse's ability to mount an early IFN- γ response (De Souza *et al.*, 1997) and early IFN- γ has been shown to limit initial parasite replication (Fell *et al.*, 1998), thereby controlling the infection until later acting responses kick in to clear the parasitemia (Langhorne *et al.*, 1998). The early versus late dichotomy most probably mirrors the innate and adaptive immune response and it therefore follows that early IFN- γ may be derived from NK and $\gamma\delta$ T cells and later IFN- γ may derive from $\alpha\beta$ T cells.

The data collected in mice is supported by studies in humans comparing individuals with varying levels of clinical immunity to malaria. IFN- γ levels have been shown to be higher in symptomatic than asymptomatic individuals (Mshana *et al.*, 1991) (Riley *et al.*, 1991) paralleled by in vitro experiments where PBMC from clinically immune individuals produce lower levels of IFN- γ than do those from unexposed donors (Rhee *et al.*, 2001) (Chizzolini *et al.*, 1990). Interestingly, unexposed donors who theoretically have no immunological memory to malaria, are able to mount a rapid and strong IFN- γ response almost immediately. Responding lymphocytes derived from naïve

adults, include T-cells which, when cultured in vitro with malaria antigen, respond in a classical MHC-restricted manner. They proliferate and produce IFN- γ in amounts comparable to true malaria memory T-cells (Dick *et al.*, 1996). Thus, naïve hosts are able to mount a strong response to malaria antigens by producing inflammatory cytokines from CD4⁺ T-cell clones that are parasite specific (Hirunpetcharat *et al.*, 1999). The theory surrounding this phenomenon is based on the priming of the immune cells by commonly administered vaccines, commonly occurring environmental antigens, or by other pathogens (Currier *et al.*, 1992). *Plasmodium* is thought to structurally mimic these antigens thereby soliciting the reaction of memory cells (Riley 1999). These cross-reactive $\alpha\beta$ T cells make significant amounts of IFN- γ , but this does not seem to protect naïve individuals; rather they seem to contribute to pathology. Exposure to malaria may induce protective immune responses by priming T cells that make IFN- γ in more appropriate sites, amounts and at a more appropriate time.

Therefore, developing clinical immunity may also hinge on the ability to downregulate the cross-reactive T cell response (which clearly does not protect naïve individuals) leaving the innate response to control initial parasitemia. The idea that the cellular sources of IFN- γ may change depending on level of immunity - which may in turn influence the absolute levels that are ultimately produced - has not been investigated to date. Given the dual role of IFN- γ , it follows that its production needs to be tightly regulated in order to simultaneously achieve clearance of infection and avoidance of detrimental clinical symptoms, a state which is characteristic of clinical immunity. In order to begin to understand how this may happen, the cellular sources of IFN- γ and their role in the acquisition of clinical immunity to malaria need to be revealed. This chapter describes a preliminary study attempting to elucidate the cellular origins of IFN- γ induced by to *P. falciparum* by **a**) taking blood samples from endemically exposed - and therefore clinically immune - Ghanaian adults and European, non-immune controls, **b**) extracting PBMC and culturing them with malaria antigens at two time points to assess the size and quality of the innate and adaptive immune responses, **c**) using flow cytometry to determine the source and intensity of the IFN- γ response, and **d**) establishing further hypotheses to be tested in future work.

3.2 Materials and methods

3.2.1 Donors

Ghanaian donors (n=13) were recruited as part of a larger study from the village of Dodowa, approximately 10 miles northeast of the capital, Accra, as described in section 2.1. Due to the large volume of blood required for PBMC separation and flow cytometry, the donor pool for this study was limited to adults and mature adolescents. The control donors were either completely malaria naïve (n=6) or had been exposed to malaria between 1-25 years previously (n=3) (Table 3.1). Parasite infection at time of sampling was determined by microscopic examination of Giemsa-stained thick blood films.

3.2.2 Cryopreservation of PBMC

Since it was not possible to perform the flow cytometry in Ghana, PBMC had to be cryopreserved and transported to LSHTM for analysis. PBMC were isolated as described in section 2.6, counted, and a maximum of 10 million cells/ 750 µl of growth medium were placed in each cryovial. 750 µl of freezing solution (55%RPMI 1640, 25% FCS (Gibco) and 20% DMSO), were added to each cryovial and then cells were placed in a motorized device which lowered them slowly into a liquid nitrogen tank. The cells were frozen in liquid nitrogen vapor at about -1°C per minute. The vials were then transported back to LSHTM in nitrogen vapor for further analysis. For consistency, for this study, cells from UK donors were cryopreserved in a similar manner.

3.2.3 Antigen Preparations and Dilutions

Prior to thawing the PBMC, the various antigens and control stimuli were diluted and plated. Live parasites (iRBC), schizont lysate (PfSL) and uninfected red blood cells (uRBC) were all used at a concentration of 1×10^5 schizonts or cells per well. A mycobacterial antigen, *M. tuberculosis* Purified Protein Derivative (PPD), was used at a concentration of 10 micrograms per ml as a classical recall antigen. Finally,

donor	Ethnicity	Country of residence	age	sex	Time since last exposure to <i>P. falciparum</i>	Parasite + at time of bleeding?
100	Af	Ghana	26	F	Endemic exp	No
101	Af	Ghana	17	F	Endemic exp	Yes
129	Af	Ghana	37	M	Endemic exp	No
157	Af	Ghana	73	F	Endemic exp	No
158	Af	Ghana	39	F	Endemic exp	No
162	Af	Ghana	22	M	Endemic exp	No
163	Af	Ghana	43	F	Endemic exp	No
164	Af	Ghana	16	M	Endemic exp	No
167	Af	Ghana	19	F	Endemic exp	Yes
173	Af	Ghana	71	F	Endemic exp	No
191	Af	Ghana	22	M	Endemic exp	Yes
194	Af	Ghana	43	M	Endemic exp	No
200	Af	Ghana	18	M	Endemic exp	Yes
Kt	Af	UK	Adult (>20)	M	25 years	No (not tested)
O	Af	UK	adult	M	11 years	No (not tested)
Gb	Af	UK	adult	M	> 1 year	No (not tested)
Ka	C	UK	adult	F	never	No (not tested)
Jc	C	UK	adult	F	never	No (not tested)
Jd	C	UK	adult	F	never	No (not tested)
Ba	C	UK	adult	F	never	No (not tested)
S	As	UK	adult	F	never	No (not tested)
Am	Af	UK	adult	F	never	No (not tested)

Table 3.1: Donor characteristics. Ghanaian and control donors used as part of the studies described in this chapter are summarized in this table. Af=African, C=Caucasian and As=Asian (Indian subcontinent).

phytohemagglutinin (PHA) (Difco) and growth medium (GM) were used as a positive and a negative control, respectively. PHA, a mitogen, was used at a concentration of 3 µg/ml.

3.2.4 PBMC Culture

Following the plating of the antigens, cells were removed from liquid nitrogen and thawed in a 37°C water bath for one minute. Each donor’s cells (from multiple cryovials) were then pooled and washed twice in 10 ml RPMI 1640 (Gibco) with 5% heat inactivated human AB serum, 100 Units/ml penicillin, and 100µg/ml streptomycin. Viable cells were then counted by Trypan blue exclusion, diluted in the correct amount of growth medium (RPMI 1640, 5% human AB serum, 100 Units/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine (Gibco BRL)) and 10⁶ cells/ml were placed in each well of a 24-well flat bottom tissue culture plate (Nunc). An average of 75% of recovered cells were viable. The cultures were then incubated at 37°C in an atmosphere of 5% carbon dioxide for 24 hours or 6 days. Since PHA is such a potent mitogen, an extra unstimulated well was added to the six day cultures and PHA was added on day 5, so that PHA responses could be analyzed after 24 hours.

3.2.5 Staining Plan

The aim of the study was to identify IFN-γ-producing CD4+ and CD8+ αβ T cells, γδ T cells, and NK cells. The marker for each population is αβTCR, γδTCR and CD56, respectively, although the CD56 marker can be found on activated T cells as well as NK cells. Therefore, cells were also stained with anti-CD3 to differentiate T cells (CD3+) from NK cells (CD56+CD3-). Likewise, the CD8 marker can be found on some γδ T cells as well as αβ T cells. Therefore, in order to correctly identify the CD8+ αβ T cells, another triple staining combination used αβ-FITC labeled antibody alongside a CD8-TRI antibody and the standard IFN-γ-PE antibody. Below is a table summarizing the different staining combinations used (Table 3.2).

Cell type	24 Hours	Stimulus					
		PH A	PPD	PfSL	uRB C	GM	
NK	Isotype control FITC/ TRI/PE	1	3	5	7	9	
	CD56-FITC/CD3-TRI/IFN- γ -PE	2	4	6	8	10	
	6 Days						
		PPD	PfS L	iRBC	uRB C	GM	PHA
CD8	Isotype control FITC/ TRI/PE	1	5	9	13	17	21
	$\alpha\beta$ -FITC/CD8-TRI/IFN- γ -PE	2	6	10	14	18	22
CD4	CD4-FITC/IFN- γ -PE	3	7	11	15	19	
$\gamma\delta$	$\gamma\delta$ -FITC/IFN- γ -PE	4	8	12	16	20	

Table 3.2: Staining plan. This table summarizes the staining combinations used to identify IFN- γ -producing cells of specific surface phenotypes. The top part of the table describes the staining after 24-hours activation and the bottom half describes the staining after 6-days activation. A total of 32 cell-staining combinations were used for each donor, 10 at 24 hours and 22 at 6 days.

3.3 Results

3.3.1 Comparison of flow cytometric indices for fresh and frozen PBMC

Due to the lack of a functioning flow cytometer at the study site, it was necessary to freeze down PBMC and transfer them to LSHTM for analysis. In order to assess the reliability of data from frozen cells, I cultured fresh and frozen cells from two control (UK) donors for six days and compared their FACS profiles. An example of PfSL stimulated cells stained with $\alpha\beta$ -FITC, CD4-FITC, and $\gamma\delta$ -FITC monoclonal antibodies, is shown in Figure 3.1. The threshold was set at 200 for the forward scatter (FSC) in order to eliminate the majority of the dead cells. In the forward and side scatter plot (FSC-SSC) of the frozen cells, an intermediate population is evident between the dead and live cells (Figure 3.1a, circled in blue), and is significantly more pronounced than for fresh cells. This was observed throughout the course of the study, however, in most donors, the effect was not as prominent as show here. Figure 3.1 shows the resulting cell percentages when this intermediate population is excluded (circled in blue-middle column) or included (circled in blue-right column). Since the resulting percentages (as Figure 3.1b,c,d) when this intermediate population was excluded resembled the fresh cell proportions more closely, it was decided to exclude this population in all further analysis of the individuals in which it occurred.

3.3.2 IFN- γ production at 24 hours

Cells were stimulated with PfSL and uRBC to assess the extent of malaria-specific IFN- γ induced after 24 hours. In an attempt to keep staining combinations at a manageable level, the iRBC antigen was not used at this time-point. All IFN- γ + cells were gated as illustrated in Figure 3.2, and the absolute number falling within this gate was recorded. Data for the 13 Ghanaian donors and the 9 controls are shown in Table 3.3. With the exception of 4 donors (158, 191, 194 and S) there were very low levels of malaria specific IFN- γ production at 24 hours, and the difference in the amount induced by PfSL versus the uRBC negative control, for each donor group, was not statistically significant (Ghanaians, Wilcoxon signed rank test, $z=0.4$, $n=13$, $p=0.7$; Controls, $z=1.4$,

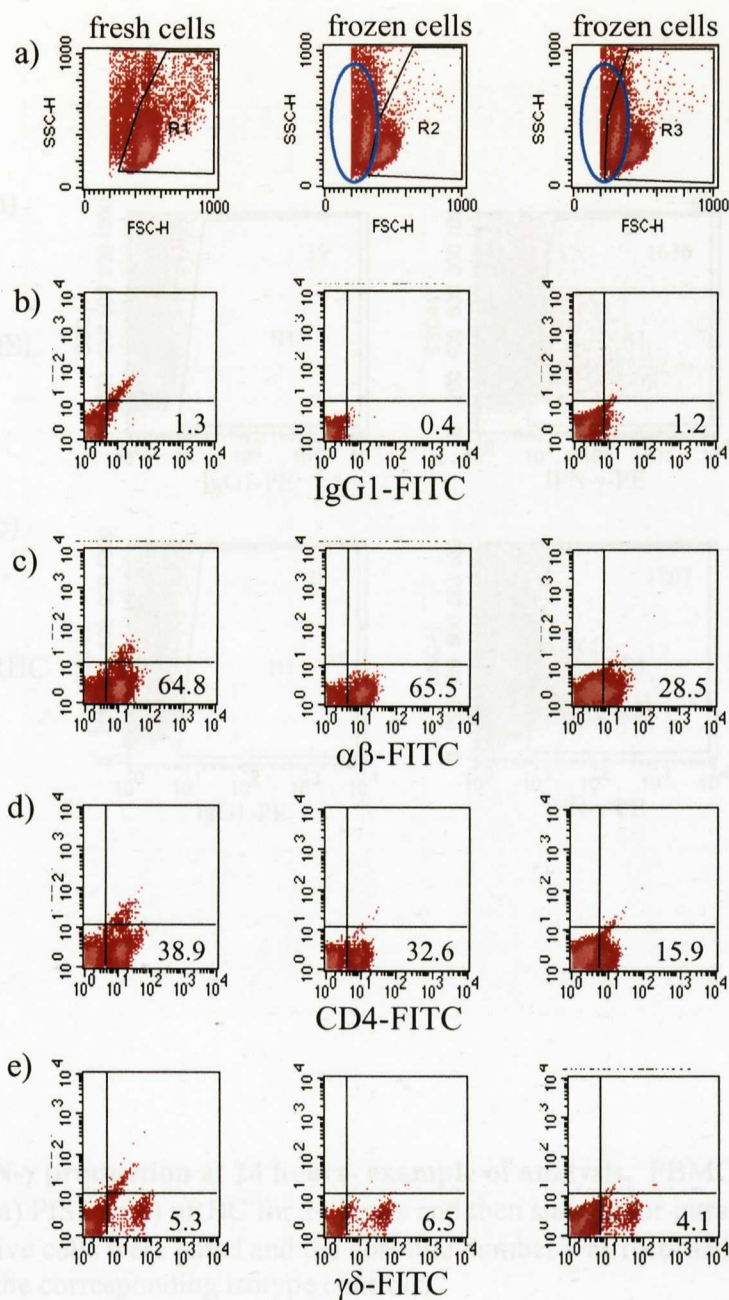


Figure 3.1: Fresh versus freeze-thawed cells. Fresh and freeze-thawed cells from the same donor were stained side by side to compare the viability and staining profiles. The left column shows fresh cells and the middle and right columns show frozen cells all stained with (b) IgG1-FITC isotype control, (c) $\alpha\beta$ -FITC, (d) CD4-FITC and (e) $\gamma\delta$ -FITC surface stains. The very top row (a) shows the forward and side scatter profiles of fresh versus freeze-thawed cells. All cells were cultured with PfSL for 6 days and a total of 30,000 events were collected. Numbers refer to the percentage of gated cells falling in the lower right quadrant. The intermediate cell population is circled in blue.

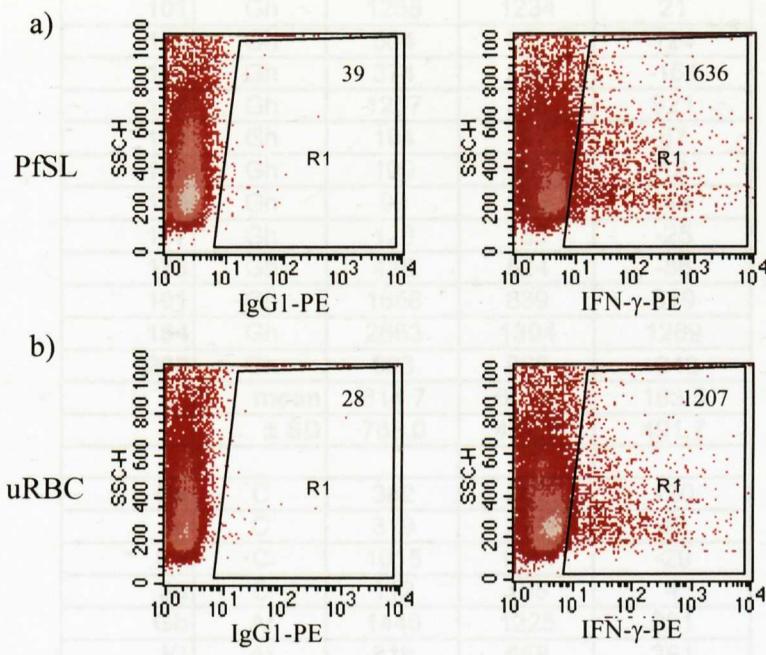


Figure 3.2: IFN- γ production at 24 hours- example of analysis. PBMC were incubated with a) PfSL or b) uRBC for 24 hours and then stained for intracellular IFN- γ . All IFN- γ positive cells were gated and the absolute number was recorded. The left column shows the corresponding isotype controls.

# IFN- γ cells/20,000 events				
donor	ethnicity	PfSL	uRBC	PfSL-uRBC
100	Gh	1279	1467	-188
101	Gh	1255	1234	21
129	Gh	504	380	124
157	Gh	374	535	-161
158	Gh	1267	346	921
162	Gh	194	127	67
163	Gh	109	97	12
164	Gh	99	143	-44
167	Gh	128	153	-25
173	Gh	438	524	-86
191	Gh	1658	839	819
194	Gh	2663	1394	1269
200	Gh	623	966	-343
	mean	814.7	631.2	183.5
	\pm SD	768.0	497.6	491.7
Ba	C	382	262	120
Jc	C	319	271	48
Ka	C	1015	1044	-29
Jd	C	112	108	4
Gb	Af	1446	1225	221
Kt	Af	819	558	261
O	Af	2143	2247	-104
Am	Af	82	136	-54
S	As	1597	1179	418
	mean	879.4	781.1	98.3
	\pm SD	728.1	709.0	171.7

Table 3.3: Number of IFN- γ positive cells at 24 hours. PBMC were incubated with PfSL or uRBC for 24 hours and analyzed as illustrated in Figure 3.2. The absolute number of IFN- γ positive PBMC per 20,000 events collected was recorded for all Ghanaian (clinically immune, numbered) and control (non-immune, lettered) donors. The far right column shows PfSL values with the uRBC negative control subtracted. Ethnicity is defined as Gh=Ghanaian, C=Caucasian, Af=African, As=Asian.

n=9, p=0.17). Differences between Ghanaian and control malaria-specific IFN- γ responses were also tested and were found not to be statistically significant (Mann-Whitney test, z=-0.2 , n=22, p=0.8).

3.3.3 NK cells in African and Caucasian donors

Since no differences were found in IFN- γ production from cells stimulated with PfSL, no further analysis was done to look at cell sources. However, resting NK cells (unstimulated) were analyzed independently of IFN- γ production for each donor, and levels were found to be higher in controls than in Ghanaians. Figure 3.3 illustrates the analysis methodology used. All CD56+ cells were gated and then examined for CD3 expression. Only cells negative for CD3 were defined as NK cells (i.e. left half of Figure 3.3c). The number of NK cells was expressed as a percentage of live lymphocytes (Figure 3.4). The percentage of lymphocytes that are CD56+CD3- NK cells was significantly lower among the Ghanaian donors (mean=0.68, standard deviation=0.63) than among the Caucasian control donors (mean=9.7, standard deviation=4.8); this difference was statistically significant (Mann-Whitney test, z=-3.9, n=22, p=0.0001). The control African donors seemed to be either “high NK” donors (i.e. like the Caucasians, n=2) or “low NK” donors (i.e. like the Ghanaian donors, n=2).

3.3.4 IFN- γ production at 6 days

By incubating PBMC for six days with the antigenic stimuli, insight can be gained into the adaptive, antigen-specific immune response to malaria as defined by IFN- γ release. Many previous studies have shown that IFN- γ production peaks 5-7 days post infection/ stimulation as measured by ELISA (Waterfall *et al.*, 1998) (Rhee *et al.*, 2001). I chose a six day time-point for this study based on our standardized lab-based protocol, in order to maintain consistency with previous work. An additional antigen, live parasites (iRBC) was included, as well as an additional set of staining combinations to identify $\gamma\delta$ T cell activity. Figure 3.5 displays how the analysis for this time-point was carried out. All IFN- γ + cells were gated and then analyzed for CD8/ $\alpha\beta$, CD4 and $\gamma\delta$ to determine the source of IFN- γ .

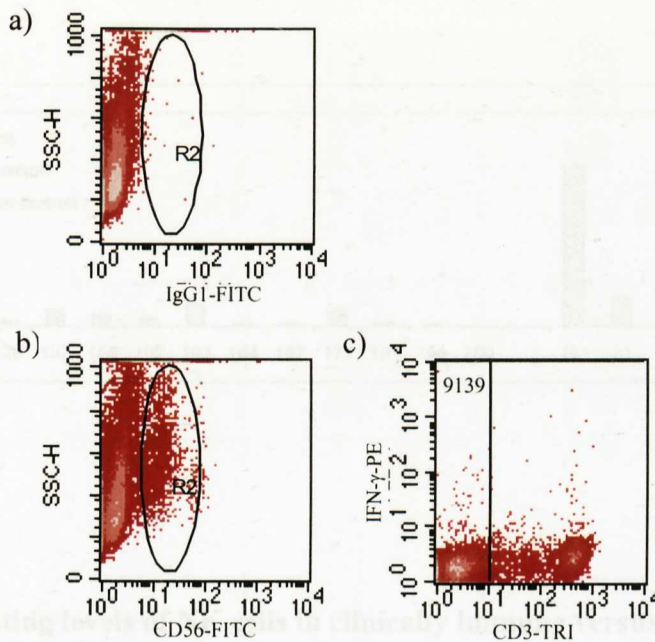


Figure 3.4: Resting PBMC from unexposed donors. PBMC were incubated for 24 hours in growth medium and analyzed for surface NK cell markers as illustrated in Figure 3.3. The proportion of NK cells was calculated as a percentage of live lymphocytes for each donor. Ghanaian, healthy female donors are shown on the left (checkered bars), African controls in the middle (striped bars) and Caucasian/Asian controls on the right (solid bars).

Figure 3.3: Absolute numbers of NK cells- example of analysis. PBMC were incubated in growth medium for 24 hours and then stained for surface CD56, CD3 and intracellular IFN- γ . Cells were gated on CD56 expression (b) and subsequently CD56+CD3- cells (both IFN- γ + and IFN- γ -) were counted to determine the resting NK cell numbers in each donor, in this case 9139/30,000 (c). The top scatter plot (a) illustrates the corresponding isotype control. The total of 30,000 events were acquired.

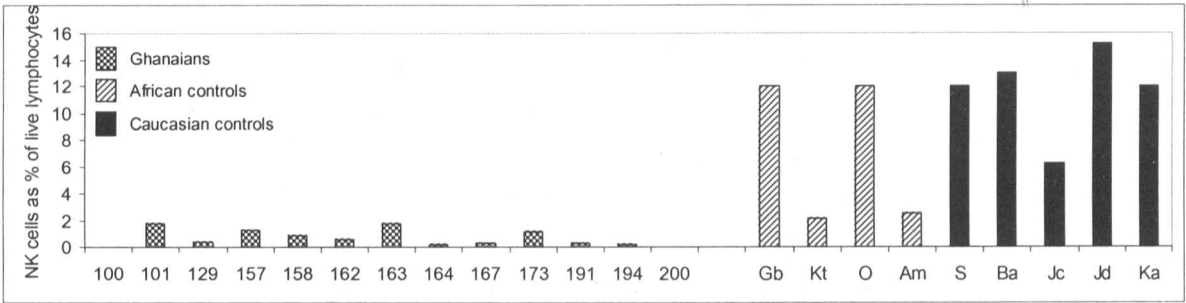


Figure 3.4: Resting levels of NK cells in clinically immune versus unexposed donors. PBMC were incubated for 24 hours in growth medium and analyzed for surface NK cell markers as illustrated in Figure 3.3. The proportion of NK cells was calculated as a percentage of live lymphocytes for each donor. Ghanaian, clinically immune donors are shown on the left (checkered bars), African controls in the middle (striped bars) and Caucasian/Asian controls on the right (solid bars).

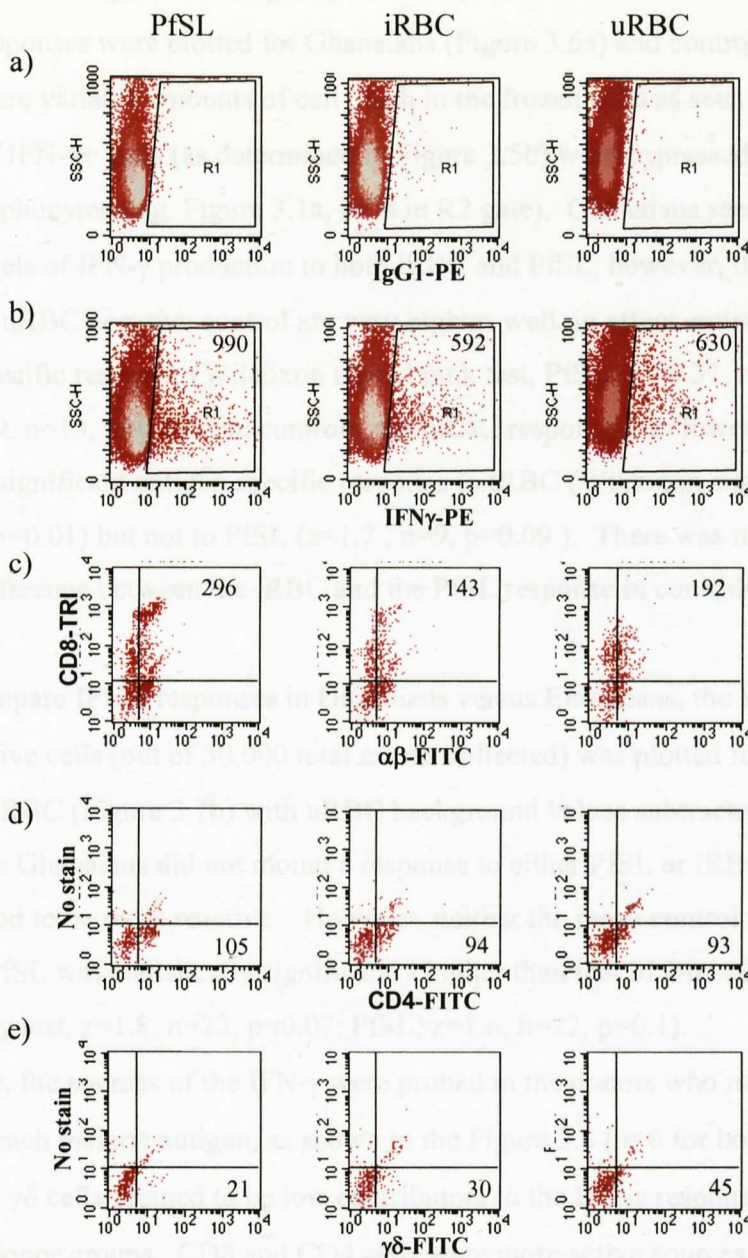


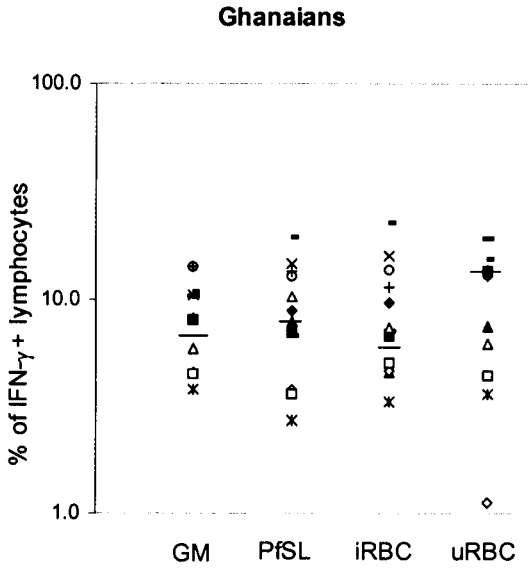
Figure 3.5: Sources of IFN- γ at 6 days - example of analysis. PBMC's (10^6 per ml) were cultured with 1×10^5 freeze-thawed schizonts (PfSL), 1×10^5 live, parasitized red blood cells (iRBC) or 1×10^5 uninfected red blood cells (uRBC). Intracellular IFN- γ and cell surface phenotype were analyzed by flow cytometry. IFN- γ^+ cells were gated (b) and analyzed for expression of TCR $\alpha\beta$ and CD8 (c), CD4 (d) or TCR $\gamma\delta$ (e). The top row (a) illustrates the corresponding isotype controls. The number of IFN- γ^+ cells per 30,000 events is shown.

In order to compare the antigen specific responses within each donor group, their total IFN- γ responses were plotted for Ghanaians (Figure 3.6a) and controls (Figure 3.6b). Since there were variable amounts of cell death in the frozen cells as seen in Figure 3.1, the number of IFN- γ + cells (as determined in Figure 3.5b) were expressed as a percentage of all live lymphocytes (e.g. Figure 3.1a, cells in R2 gate). Ghanaians seemed to have fairly high levels of IFN- γ production to both iRBC and PfSL, however, the background (GM) and the uRBC negative control are very high as well, in effect entirely eliminating the antigen specific response (Wilcoxon signed rank test, PfSL: $z=-0.31$, $n=13$, $p=0.75$; iRBC: $z=-0.39$, $n=13$, $p=0.69$). In controls, the uRBC response was lower, and there was a statistically significant antigen specific response to iRBC (Wilcoxon signed rank test, $z=2.49$, $n=9$, $p=0.01$) but not to PfSL ($z=1.7$, $n=9$, $p=0.09$). There was no statistically significant difference between the iRBC and the PfSL response in controls ($z=1.7$, $n=9$, $p=0.09$).

To compare IFN- γ responses in Ghanaians versus Europeans, the absolute number of IFN- γ positive cells (out of 30,000 total events collected) was plotted for PfSL (Figure 3.7a) and for iRBC (Figure 3.7b) with uRBC background values subtracted. The majority of the Ghanaians did not mount a response to either PfSL or iRBC, whereas the controls seemed to be more reactive. However, neither the mean control response to iRBC nor to PfSL was statistically significantly higher than that of Ghanaians (iRBC, Mann-Whitney test, $z=1.8$, $n=22$, $p=0.07$; PfSL, $z=1.6$, $n=22$, $p=0.1$).

Finally, the sources of the IFN- γ were probed in the donors who mounted an IFN- γ response to each malaria antigen, as shown in the Figure 3.8 ($n=6$ for both Ghanaians and controls). $\gamma\delta$ cells seemed to be low contributors to the IFN- γ response for both antigens and donor groups. CD8 and CD4 cells were more active sources of IFN- γ than $\gamma\delta$ T cells, and CD4 cells seemed to be slightly more efficient at producing the cytokine, particularly in controls. None of the differences between CD8 and CD4 IFN- γ production were statistically significant (Wilcoxon signed rank test, Ghanaians, PfSL, $z=1.8$, $n=6$, $p=0.08$; Ghanaians, iRBC, $z=-1.2$, $n=6$, $p=0.2$; controls, PfSL, $z=-0.5$, $n=6$, $p=0.6$; controls, iRBC, $z=0.9$, $n=6$, $p=0.3$).

a)



b)

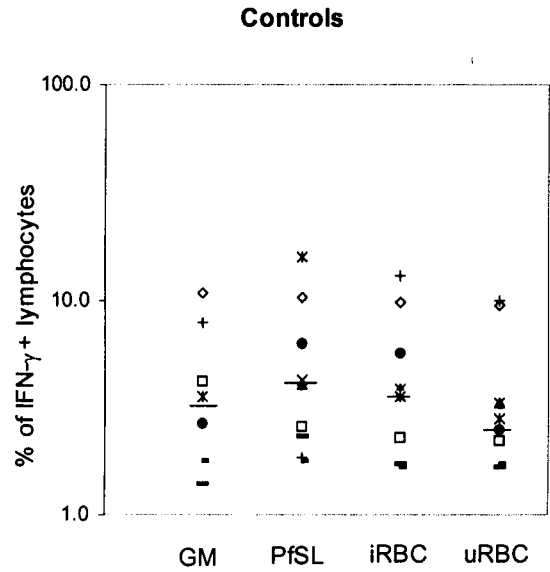


Figure 3.6: Percentage of IFN- γ positive cells after 6 days. PBMC's (10^6 per ml) were cultured with 1×10^5 freeze-thawed schizonts (PfSL), 1×10^5 live, parasitized red blood cells (iRBC) or 1×10^5 uninfected red blood cells (uRBC). Intracellular IFN- γ and cell surface phenotype were analyzed by flow cytometry as illustrated in Figure 3.5. The percent of IFN- γ + cells (calculated as a percentage of all live lymphocytes) for each Ghanaian donor (a) and each control donor (b) are plotted on a log scale for each of the three antigen preparations.

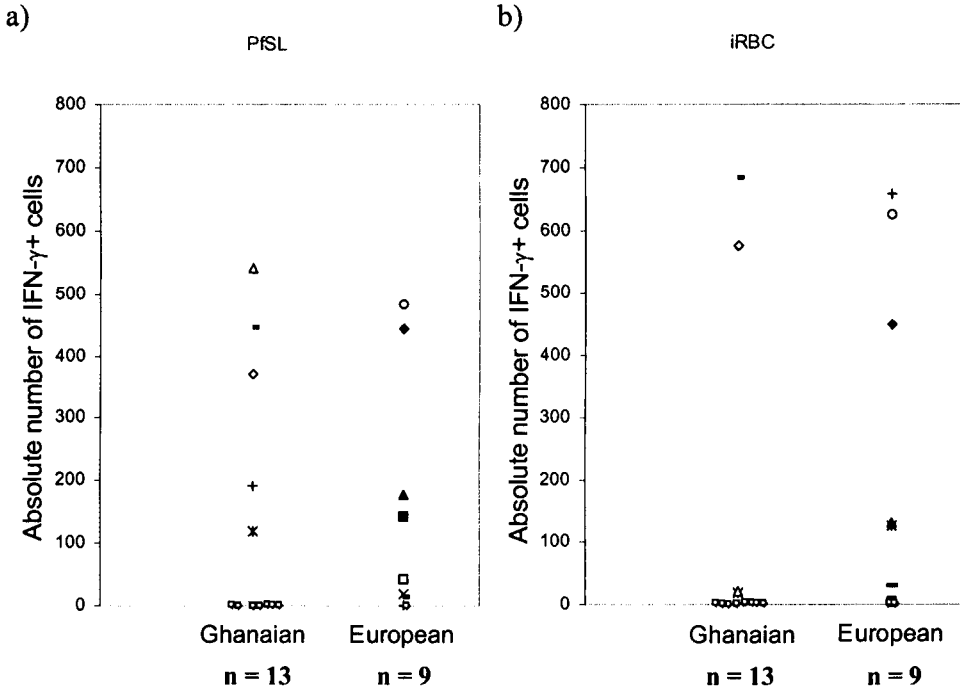


Figure 3.7: Ghanaian versus control IFN- γ response to PfSL and iRBC after 6 days. PBMC's (10^6 per ml) were cultured with 1×10^5 freeze-thawed schizonts (PfSL), 1×10^5 live, parasitized red blood cells (iRBC) or 1×10^5 uninfected red blood cells (uRBC). Intracellular IFN- γ and cell surface phenotype were analyzed by flow cytometry as illustrated in Figure 3.5. Numbers of IFN- γ cells to PfSL (a) and to iRBC (b) with uRBC control values already subtracted, are plotted for both Ghanaians and controls. The horizontally plotted circles on the x-axis indicate the number of donors with no IFN- γ positive cells.

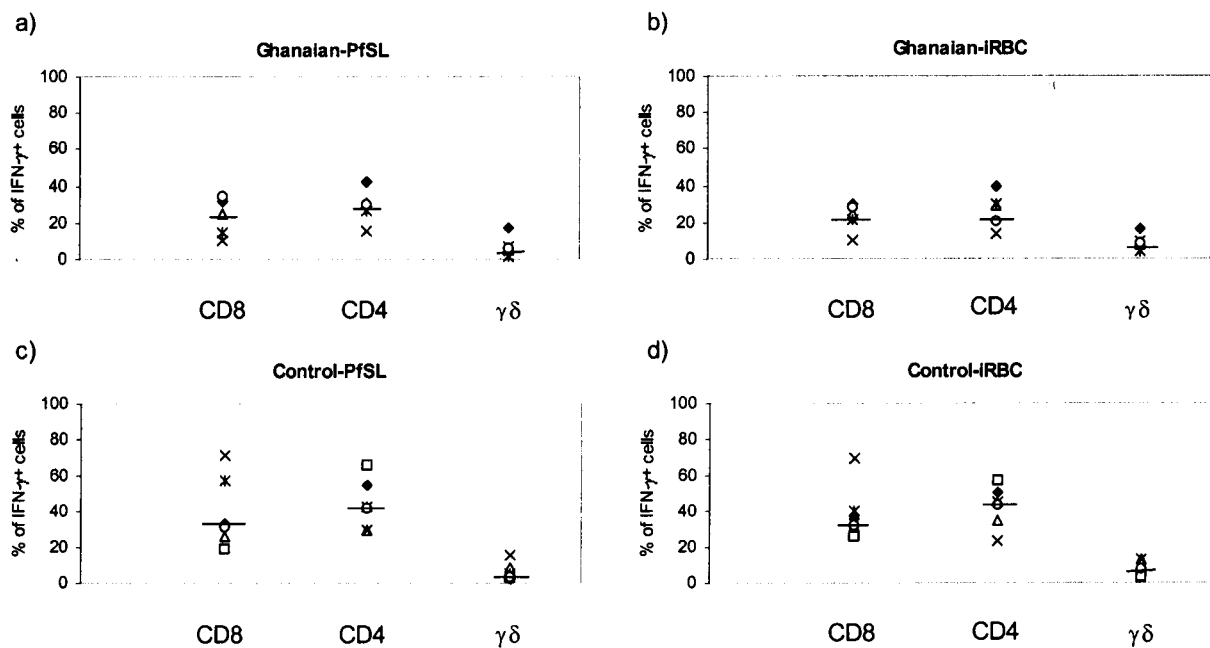


Figure 3.8: Sources of IFN- γ after 6 days. PBMC's (10^6 per ml) were cultured with 1×10^5 freeze-thawed schizonts (PfSL), 1×10^5 live, parasitized red blood cells (iRBC) or 1×10^5 uninfected red blood cells (uRBC) for 6 days. Intracellular IFN- γ was stained for in combination with CD4, CD8 or $\gamma\delta$ TCR cell surface phenotypes and analyzed by flow cytometry as illustrated in Figure 3.5. The percentage of the total IFN- γ positive cells belonging to each phenotype was calculated and plotted for the Ghanaian response to PfSL (a) and to iRBC (b) and the control response to PfSL (c) and to iRBC (d).

3.4 Discussion

The objective of this study was to investigate the cellular sources of IFN- γ in response to *P. falciparum* infection using in vitro stimulation of PBMC with malaria antigens. The preferential activation of CD4⁺ cells into blasts as opposed to CD8⁺ cells has been well documented in response to PfSL (Goodier *et al.*, 1995), (Waterfall *et al.*, 1998), (Dick *et al.*, 1996). However, $\gamma\delta$ cells have been found to be most effectively stimulated by live parasites, namely the parasitic components that are released during the process of schizont rupture (Waterfall *et al.*, 1998) (Goodier *et al.*, 1995). It is possible that live schizonts prompt the general release of IL-2, which in turn is able to significantly activate the $\gamma\delta$ cell population. Also, research has shown that different cell types are preferentially stimulated to produce IFN- γ by different antigenic components of the malaria parasite (Waterfall *et al.*, 1998).

The initial part of the study involved collection and freezing of PBMC from Ghanaian donors. This process was carried out following the protocol developed by the immunology team at the Noguchi Institute in Ghana. The reliability of freeze-thawing cells and their subsequent viability have been well documented by previous studies comparing them to fresh cells (Bell *et al.*, 1972), (Merker *et al.*, 1979), (Wood *et al.*, 1972). Since it was impossible to assay the cells at the study site in Ghana, freezing the cells provided a workable alternative and enabled me to assess these individuals' IFN- γ response to *P. falciparum* through flow cytometry. Regardless of how successful this technique has been in the past, it is impossible to exclude the possibility that the resulting differences in cell numbers are due to a selective depletion of specific cell types making others appear more active/elevated. Indeed, the FCS-SSC profile of fresh and frozen cells was different with intermediate population of cells on the FSC-SSC plots of the frozen PBMC, which were believed to be dying cells. This indicates some potential for skewing the results. In order to rule out these potential confounders it was deemed preferable if further studies were done exclusively on fresh cells. However, these experiments were not designed to give a categorical answer to the question of IFN- γ origin and regulation, but rather to probe the differences between the naïve and clinically

immune responses and provide more specific queries to follow up. This aim was successfully accomplished, in that certain trends became apparent in the analysis which provided a point of departure for future work.

Innate response

The data collected at the 24 hour time-point, reflecting the innate response, revealed that dead schizont lysate does not induce a noteworthy IFN- γ response early on, as compared with that induced by the uRBC negative control. This observation held true for all donors regardless of their state of immunity or exposure. These results may indicate one of two possible scenarios, namely either that there is no rapid/innate response to *P. falciparum* or that dead parasites are unable to induce IFN- γ at such an early time-point. Since previous studies have shown differences in the lymphoproliferative and cytokine responses induced by different parasite components (Riley *et al.*, 1988), and more notably between live and dead parasites (Waterfall *et al.*, 1998) (Hensmann *et al.*, 2001), this topic requires further investigation and is addressed in the next chapter.

The most interesting observation gleaned from the 24-hour data was that the Caucasians in the control group had higher percentages of resting NK cells compared with the Ghanaians. It was hypothesized that this difference could be attributed to genetic and/or environmental determinants. The African, UK-resident controls, who could have potentially shed light on this question, were split equally between high and low NK cell percentages, therefore it was not possible to draw any conclusions on the matter. Contrary to the data outlined here, a previous study which also analyzed the NK cell composition of PBMC, in this case from Kenyans and Caucasians, as part of a study of NK responses to malaria found no difference in the percentages of CD56+ cells between these groups (Orago *et al.*, 1991).

However, the question of whether or not the differences in NK percentages seen are real or an artefact of differences in other cell percentages remains. That is to say, the Ghanaian donors could possibly have higher numbers of T cells making the NK percentages appear lower. The only way to settle this question would be by relating the percentages found back to whole white blood cell counts, data that unfortunately was not

collected on all donors. Assuming the differences are indeed real further studies are needed of NK cells in Caucasians and malaria exposed and unexposed Africans.

Adaptive response

The 6-day assay yielded higher levels of IFN- γ + cells in response to malaria antigens than the 24-hour assay. No statistically significant differences between the Ghanaians' and the controls' responses to the malaria antigens were detected, although the iRBC induced response in controls was visibly higher and may have reached statistical significance had the sample size been larger. Among the Ghanaian donors, no malaria-specific IFN- γ responses were detected. However, the background uRBC and GM values were very high, suggesting that this result is due to very high spontaneous IFN- γ release. Cryopreserved cells have previously been shown to manifest higher background cytokine production than fresh ones (D. Dodoo, personal communication) which may, in part, be accountable for the results obtained from the Ghanaians. The alternative explanation would be that the Ghanaian donors have no malaria-specific response.

In the control group, there was clear evidence of a malaria-specific response, particularly for the live parasite antigen, an observation which supports previous data (Rhee *et al.*, 2001). The predominant source seemed to be $\alpha\beta$ T cells, particularly the CD4+ subtype, with $\gamma\delta$ T cells contributing to a lower extent. Previous studies which focused on the blasting cell population in response to malaria stimulation found that the majority of blasts were CD4+ (Dick *et al.*, 1996). The results from this study focused on all IFN- γ + cells and revealed a roughly equal contribution from CD4+ and CD8+ T cells. The combination of these data seems to indicate that IFN- γ derives from both CD4 and CD8 T cells, but that cell proliferation may be limited to the CD4+ group.

This study was designed to highlight points of interest that could serve as a springboard for future work and in this sense, it was successful. The main point of interest which is delved into in the following chapters is the innate immune response, and more specifically the contribution of NK cells to inflammatory cytokine release. This study also elucidates certain experimental flaws which were dealt with in further work. Namely, the acquisition of events needs to be increased beyond the 30,000 cells collected

in this study in order to accurately enumerate the number of cells present at low frequency. Cells also need to be cultured immediately, bypassing liquid nitrogen freezing, and donors need to be grouped more stringently according to their malaria exposure. In addition, the effect of live parasites (i.e. iRBC) needs to be evaluated for early responses.

Chapter 4 Kinetics and Sources of IFN- γ Induced by *P. falciparum*

4.1 Introduction

The data in the previous chapter, revealed variations in IFN- γ responses induced by different antigen preparations, namely live versus dead parasites. These results also exposed distinct cell subsets as the respective sources of the IFN- γ , dependent upon antigen preparation as well as length of incubation, suggesting that the inflammatory cytokine response to malaria is multi-faceted, deriving from several different cell types. Several studies have shown that PBMC from malaria-naïve donors can produce IFN- γ in response to stimulation by either live or dead schizont antigens (Currier *et al.*, 1992; Dick *et al.*, 1996; Waterfall *et al.*, 1998; Zevering *et al.*, 1992). Live parasites induce proliferation of both $\alpha\beta$ and $\gamma\delta$ TCR⁺ T cells whereas dead schizont extract activates only TCR $\alpha\beta$ ⁺ T cells (Waterfall *et al.*, 1998). These cells have been widely assumed to be the major source of IFN- γ (Dick *et al.*, 1996; Goodier *et al.*, 1995). Activation of TCR $\alpha\beta$ ⁺ T cells has been ascribed to reactivation of a polyclonal population of memory cells primed by exposure to cross-reactive antigens (Currier *et al.*, 1992; Fell *et al.*, 1996; Goodier *et al.*, 1997) whereas the TCR $\gamma\delta$ ⁺ T cell response is restricted to the V γ 9V δ 2 subset (Behr *et al.*, 1992; Goerlich *et al.*, 1991), and is induced by small, phosphorylated non-protein antigens similar to those described for mycobacteria (Behr *et al.*, 1996). These findings are consistent with cytokine responses to malaria antigens in unexposed donors being derived from cells of the adaptive immune system.

However, Scragg *et al* (Scragg *et al.*, 1999) have reported very early induction of TNF- α , IL-12 and IFN- γ (within 10 hours) from PBMC by live, parasitized erythrocytes and Hensmann *et al* (Hensmann *et al.*, 2001) have shown that live parasites induce TNF- α and IFN- γ from V γ 9⁺ T cells and TNF- α from CD14⁺ monocytes, within 18 hours. Cytokine induction is dependent on the presence of both monocytes and lymphocytes (Scragg *et al.*, 1999) indicating that this is not a classical endotoxin-like response as had previously been thought (Schofield *et al.*, 1993). Other innate cellular involvement in the

inflammatory response to malaria, such as that of NK cells, has not been studied thoroughly. The potential NK cell contribution to malaria-induced IFN- γ production has been somewhat marginalized in favor of their reported cytotoxic activity against *P. falciparum*-infected erythrocytes (Ojo-Amaize *et al.*, 1981) (Stach *et al.*, 1986) (Theander *et al.*, 1987).

The purpose of the experiments described in this chapter, therefore, was to investigate the kinetics and cellular origins of IFN- γ induced by *P. falciparum* malaria in naïve and malaria-exposed human blood donors, in order to determine the contribution of cells of the innate and adaptive immune response at different time points after activation.

4.2 Materials and Methods

PBMC were obtained from malaria exposed and unexposed donors at LSHTM or from HTD (as described in section 2.1). HTD donors had been previously diagnosed with, and successfully treated for, acute *P. falciparum* malaria approximately one month prior to blood sampling. All donors are listed in Table 4.1 with relevant characteristics.

PBMC were cultured with live *P. falciparum* infected erythrocytes (iRBC), *P. falciparum* schizonts lysate (PfSL), uninfected erythrocytes (uRBC) or PHA (as a positive control stimulation) for up to 6 days. Cell culture supernatants were collected and tested for secreted IFN- γ by ELISA (as described in section 2.9). Cells were washed, stained for surface markers and intracellular cytokines and analyzed by flow cytometry (as described in section 2.8).

N.B: Important differences between this methodology and that of Chapter 3 are the following:

- i) fresh cells were used as opposed to freeze-thawed ones
- ii) live parasitized erythrocytes (iRBC) were used
- iii) parasite antigens were used at 3×10^6 /ml rather than 1×10^5 /ml
- iv) 100,000 events were acquired from each FACS sample as opposed to 30,000

Donor	Ethnic Origin	Prior Malaria Exposure	Sex
KAT	C	N	F
016F	C	N	F
014F	C	N	F
SB	As	N	F
053M	C	N	M
TH	C	N	F
094M	As	N	M
JR	C	N	M
095F	C	N	F
073F	C	N	F
PC	C	N	M
093F	C	N	F
AJH	C	N	F
RaT	As	N	F
MH	C	N	M
BdS	As	N	M
CS	C	N	F
AMSP	Af	N	F
KT	Af	Y	M
FMO	Af	Y	M
JET	C	Y	M
HT	Af	Y	M
HTD1	Af	Y	M
HTD3	Af	Y	F
HTD4	Af	Y	M
HTD5	Af	Y	M
HTD6	Af	Y	F
HTD7	Af	Y	M
HTD8	Af	Y	M
HTD9	Af	Y	F

Table 4.1: Donor characteristics. Consenting, adult donors were recruited either from LSHTM or HTD. This table summarizes their characteristics including ethnicity (C=Caucasian, As=Asian, Af=African), previous malaria exposure and sex.

4.3 Results

4.3.1 IFN- γ response to *P. falciparum* by cells from malaria-naïve donors

We examined the kinetics of IFN- γ secretion from PBMC of 5 European, malaria naïve donors in response to iRBC or PfSL, measuring IFN- γ concentration in culture supernatants by ELISA (Figure 4.1). Uninfected RBC (uRBC) induced minimal IFN- γ production over a period of 6 days (4.1a). Lysed parasites (PfSL) induced modest, but statistically significant IFN- γ responses in cells from all donors (4.1b). Cells from three donors produced IFN- γ from about day 4, cells from one donor made only a modest and transient response and cells from one other donor made high levels of IFN- γ within 24 hours. In contrast, live parasites (iRBC) rapidly induced high levels of IFN- γ production from cells from all donors (4.1c), with IFN- γ concentrations in excess of 10,000 pg/ml by day 4 and concentrations of up to 200,000 pg/ml by day 6. Differences between PfSL-induced and iRBC-induced IFN- γ concentrations were statistically significant at all time points (Wilcoxon signed rank test, $Z = -2.023$, $n = 5$, $P = 0.043$). Thus, the IFN- γ response to iRBC was more rapid, and maximal concentrations were 10 fold higher, than the response to PfSL. These data indicate that iRBC, but not PfSL, induces a rapid IFN- γ response (<24 hours).

4.3.2 Cellular sources of IFN- γ induced by *P. falciparum*

In order to determine which cells were making IFN- γ at different stages of the response, cytokine kinetics were examined by FACS, looking at specific cell populations to see when, and to what extent, each population was activated to synthesize IFN- γ . To determine the percentage of each cell type that is producing IFN- γ , all $\alpha\beta^+$, $\gamma\delta^+$ or CD56 $^+$ cells were gated (R1) and then analyzed separately for IFN- γ staining (and for CD3 staining in the case of the NK cells) (Figure 4.2). The number of cells staining for the isotype control (first row) was subtracted from each quadrant and the percent IFN- γ positive cells of each cell phenotype was calculated. Results from six donors are summarized in Figure 4.3.

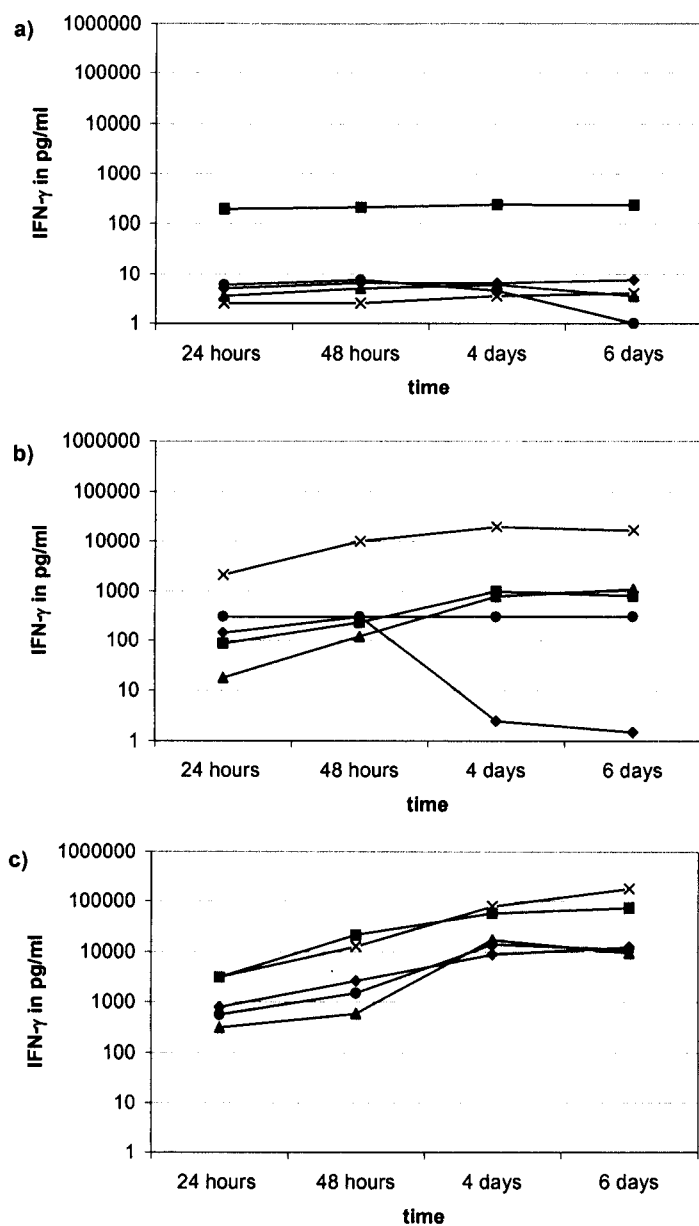
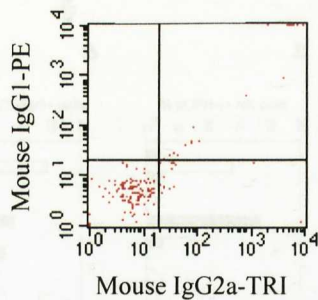
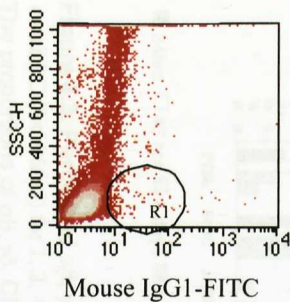


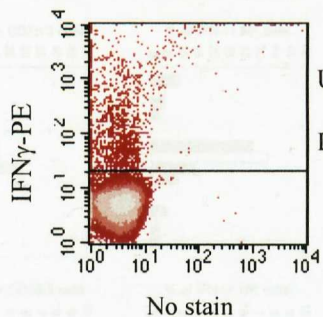
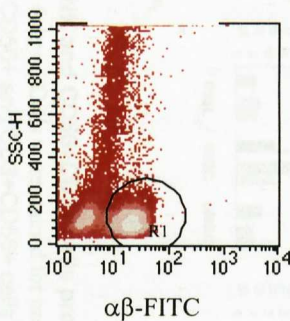
Figure 4.1: IFN- γ kinetics as measured by ELISA. PBMC's (10^6 per ml) were cultured with (a) 3×10^6 uninfected red blood cells (uRBC), (b) 3×10^6 freeze-thawed schizonts (PfSL) or (c) 3×10^6 live, parasitized red blood cells (iRBC) for up to 6 days. Cell supernatants were collected at intervals and assayed for IFN- γ by ELISA. Values for uRBC-stimulated cultures have been subtracted from values for iRBC and PfSL-stimulated cultures. Data from 5 donors are shown, each donor represented by a different symbol.

Figure 4.2: Example of flow cytometric analysis for IFN- γ production by $\alpha\beta$, $\gamma\delta$, NK and CD56+CD3+ cells.

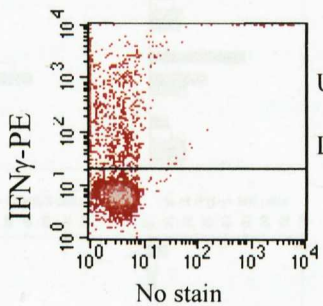
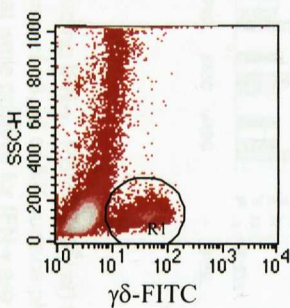
PBMC's (10^6 per ml) were cultured with 3×10^6 live, parasitized red blood cells (iRBC) for 24 hours. Intracellular IFN- γ and cell surface phenotype were analyzed by flow cytometry. Isotype control data are shown in the top row. TCR $\alpha\beta$ + cells (2nd row), TCR $\gamma\delta$ + cells (3rd row) and CD56+ cells (bottom row) were gated and analyzed for expression IFN- γ and, in the case of NK cells, CD3 (bottom row). A total of 100,000 events were analyzed. The percent of each cell phenotype producing IFN- γ was calculated by first subtracting background events (top row) and subsequently dividing the number of double positive cells by the total number of cells of a given phenotype.



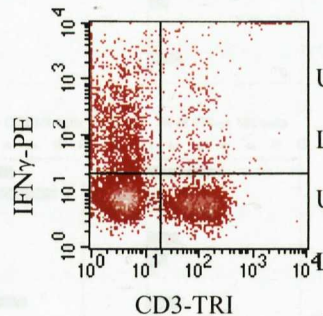
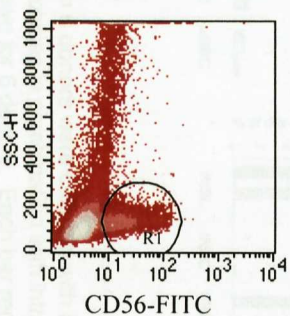
UL 1
UR 27 } 28
LL 52
LR 13 } 65



UL 1353-28= 1325
LL 62718-65= 62653 } $1325/(1325+62653)= 2.1\%$
of $\alpha\beta$ cells are IFN- γ +



UL 900-28= 872
LL 2451-65= 2386 } $872/(872+2386)= 26.8\%$
of $\gamma\delta$ cells are IFN- γ +



UL 1513-1= 1512
LL 3104-52= 3052
UR 182-27= 155
LR 1144-13= 1131 } $1512/(1512+3052)= 33.1\%$
of NK cells are IFN- γ +

$155/(155+1131)= 12.1\%$
of CD56+CD3+ cells are IFN- γ +

Donor:

$\alpha\beta$ cells

$\gamma\delta$ cells

NK cells

CD56+CD3+ cells

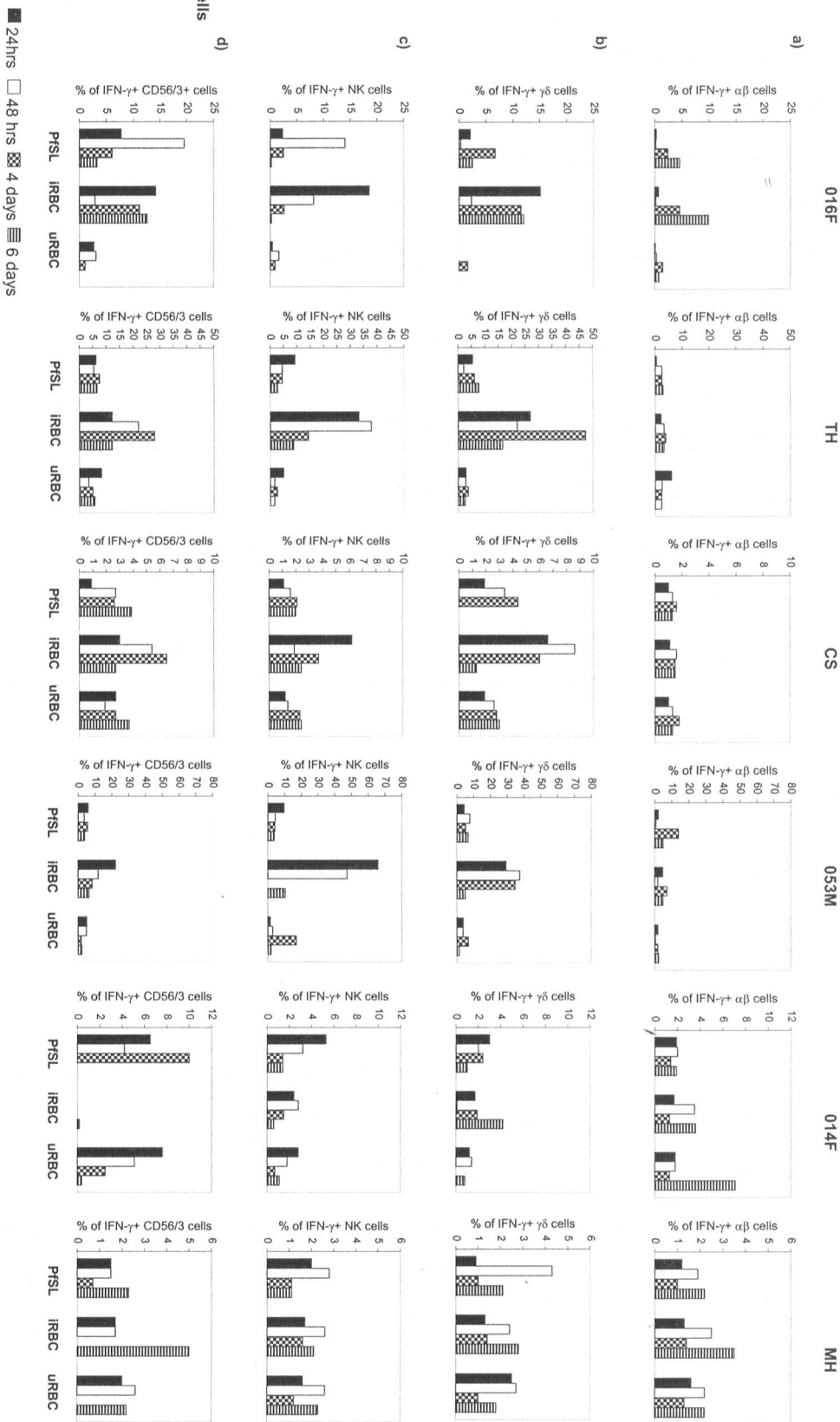


Figure 4.3: Percent of $\alpha\beta$, $\gamma\delta$, NK and CD56+CD3+ cells producing IFN- γ . PBMC (10^6 /ml) from 6 donors were cultured with PSL, iRBC or uRBC (3×10^6 /ml) for 1, 2, 4 or 6 days. Cells were stained for presence of $\alpha\beta$, $\gamma\delta$ or NK surface phenotypes in combination with intracellular IFN- γ . The proportions of $\alpha\beta$, $\gamma\delta$, CD3-CD56+ and CD3+CD56+ cells that were positive for IFN- γ are shown for 6 donors. Each bar represents a different time-point.

As seen with the ELISA analysis, iRBC seems to be better than PfSL at inducing an IFN- γ response across all cell types, however the kinetics of the response of each cell population seem to be slightly different. The $\alpha\beta$ T cells are not consistently activated to produce IFN- γ (4.3a), but in the donors where they do produce above-background levels of IFN- γ (donors MH, 014F and 016F), the response is not detectable until 4-6 days. In contrast, IFN- γ synthesis by $\gamma\delta$ T cell is seen to a greater or lesser extent in 5 out of 6 donors (the exception being MH) and occurs at any time from 24 hours to 6 days (4.3b). NK cell responses are also variable between donors, but in those donors where an NK response is seen (016F, TH, CS, 053M) it occurs within 24 hours (4.3c). CD56⁺ CD3⁺ cells also synthesize IFN- γ , reaching maximal activation between the 4th and 6th day of incubation. These cells could either represent NKT cells or activated T cells which have upregulated the CD56 marker on their surface. Without typing the α and β chains of the T cell receptor, it is impossible to categorize this group further. However, considering that the kinetics of their IFN- γ synthesis closely resembles that of the $\gamma\delta$ and $\alpha\beta$ T cells, it is logical to hypothesize that they are activated T cells (either $\alpha\beta$ ⁺ or $\gamma\delta$ ⁺) rather than innately activated NKT cells. Interestingly, IFN- γ responses by the different cell types seems to be correlated within a single donor such that donors who make a strong IFN- γ NK response at 24 hours are likely also to make strong $\gamma\delta$ T cell and CD56⁺CD3⁺ responses (e.g. 016F, TH, CS, 053M) whereas donors who made negligible NK responses (014F and MH) also made very low $\gamma\delta$ and CD56⁺ CD3⁺ responses.

4.3.3 Which cells are the major source of IFN- γ ?

Since an early burst of IFN- γ could potentially be critical in influencing the course of infection and disease, and since all cell types examined seemed to be capable of producing IFN- γ , the data were re-analyzed to determine what percentage of the total IFN- γ response could be attributed to each cell type at each time point. In this case, IFN- γ ⁺ cells were gated and this population was analyzed for expression of CD3, TCR $\alpha\beta$, TCR $\gamma\delta$ and CD56.

As an example, data for one donor, after 24 hours incubation of PBMC with 3×10^6 iRBC or uRBC or 3×10^6 lysed infected erythrocytes (PfSL) are shown in Figure 4.4. For cells incubated with iRBC, IFN- γ was derived from a mixed population of cells of which the most numerous are CD56⁺CD3⁻ NK cells. After subtraction of the background counts for the isotype control antibody and uRBC control, 62% of the iRBC-induced IFN- γ ⁺ cells were CD3⁻ CD56⁺ NK cells, the remainder were CD3⁺ T cells. After 24 hours incubation with PfSL the number of cells staining for IFN- γ was almost four-fold lower than for iRBC; after subtraction of background staining, 42% of the IFN- γ ⁺ cells were found to be NK cells.

The proportion of IFN- γ ⁺ cells belonging to each cell type is shown, for 6 donors, in Figure 4.5. Data are shown for cells activated with PfSL or iRBC for up to 6 days. As in Figure 4.3, the donors seem to fall into two groups, with donors 016F, TH, 053M and CS making an early (24 hour) response and donors MH and 014F making very little IFN- γ at 24 hours. For iRBC, in those donors making an early response, NK cells were the dominant IFN- γ ⁺ cell type at 24 hours. The NK cell response waned rapidly and by 4-6 days $\gamma\delta$ and $\alpha\beta$ T cells were the predominant source of IFN- γ . A similar, but less consistent, pattern was seen for PfSL activation with CD56⁺CD3⁺ T cells making a more obvious contribution to IFN- γ production. For the low responding donors (MH and 014F) $\alpha\beta$ ⁺ or CD3⁺CD56⁺ cells were the major source of IFN- γ at all time points, but as the total percentage of IFN- γ ⁺ cells was very low (less than 0.2% at all time points) these data are likely to be extremely inaccurate.

4.3.4 IFN- γ production by NK cells

The initial data analysis (Figures 4.3 and 4.5) indicated that a major proportion of the cells producing IFN- γ in response to live, intact parasitized RBC at 24 hours were NK cells. As NK cells are part of the innate response and are rapidly activated, we hypothesized that these cells could represent an important component of the very early response to malaria and might therefore be important in controlling the first wave of parasitemia. To determine whether rapid induction of IFN- γ from NK cells by iRBC was a universal phenomenon, we looked at the 24 hour IFN- γ response in a cohort of 30

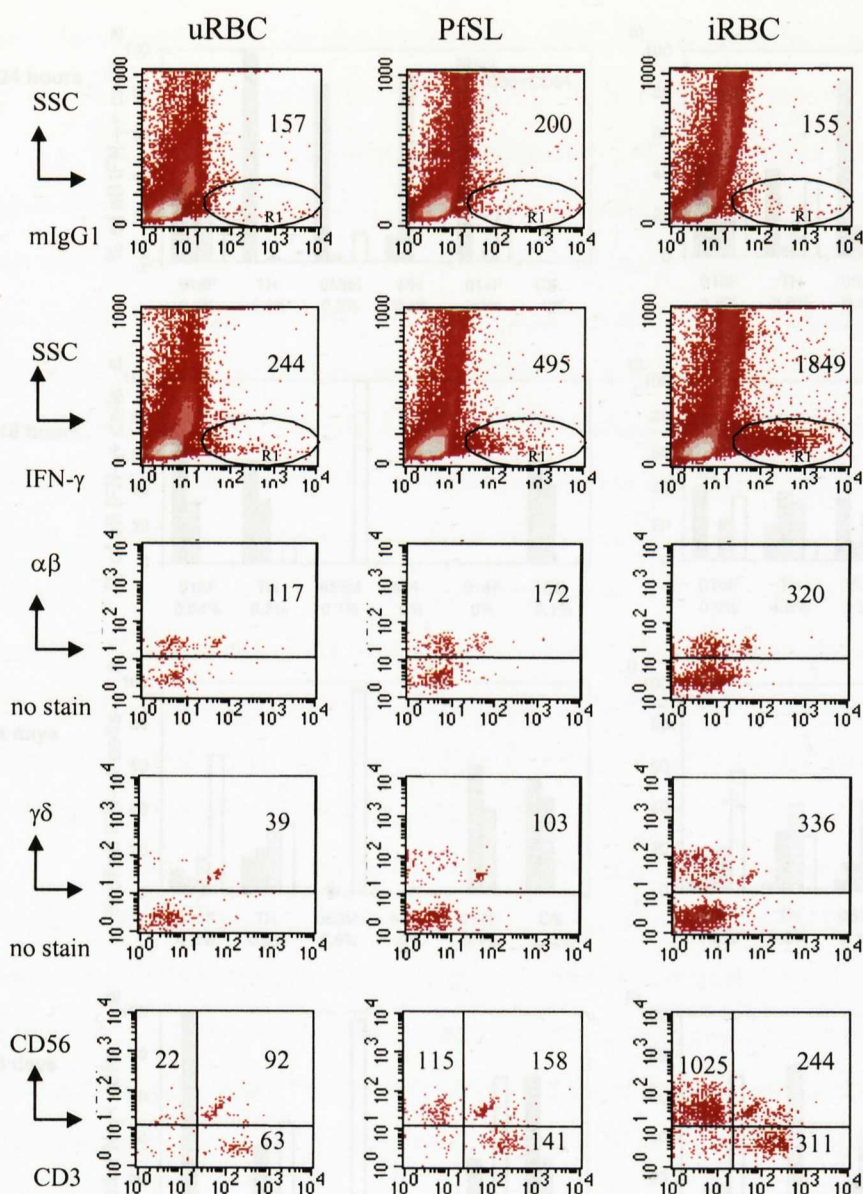


Figure 4.4: Cellular source of IFN- γ after 24 hours. PBMC's (10^6 per ml) were cultured with 3×10^6 uninfected red blood cells (uRBC), 3×10^6 freeze-thawed schizonts (PfSL) or 3×10^6 live, parasitized red blood cells (iRBC) for 24 hours. Intracellular IFN- γ and cell surface phenotype were analyzed by flow cytometry. Isotype control data are shown in the top row. IFN- γ^+ cells were gated (second row) and analyzed for expression of TCR $\alpha\beta$ (3rd row), TCR $\gamma\delta$ (4th row) and CD56/CD3 (bottom row). The number of IFN- γ^+ cells per 100,000 events was counted.

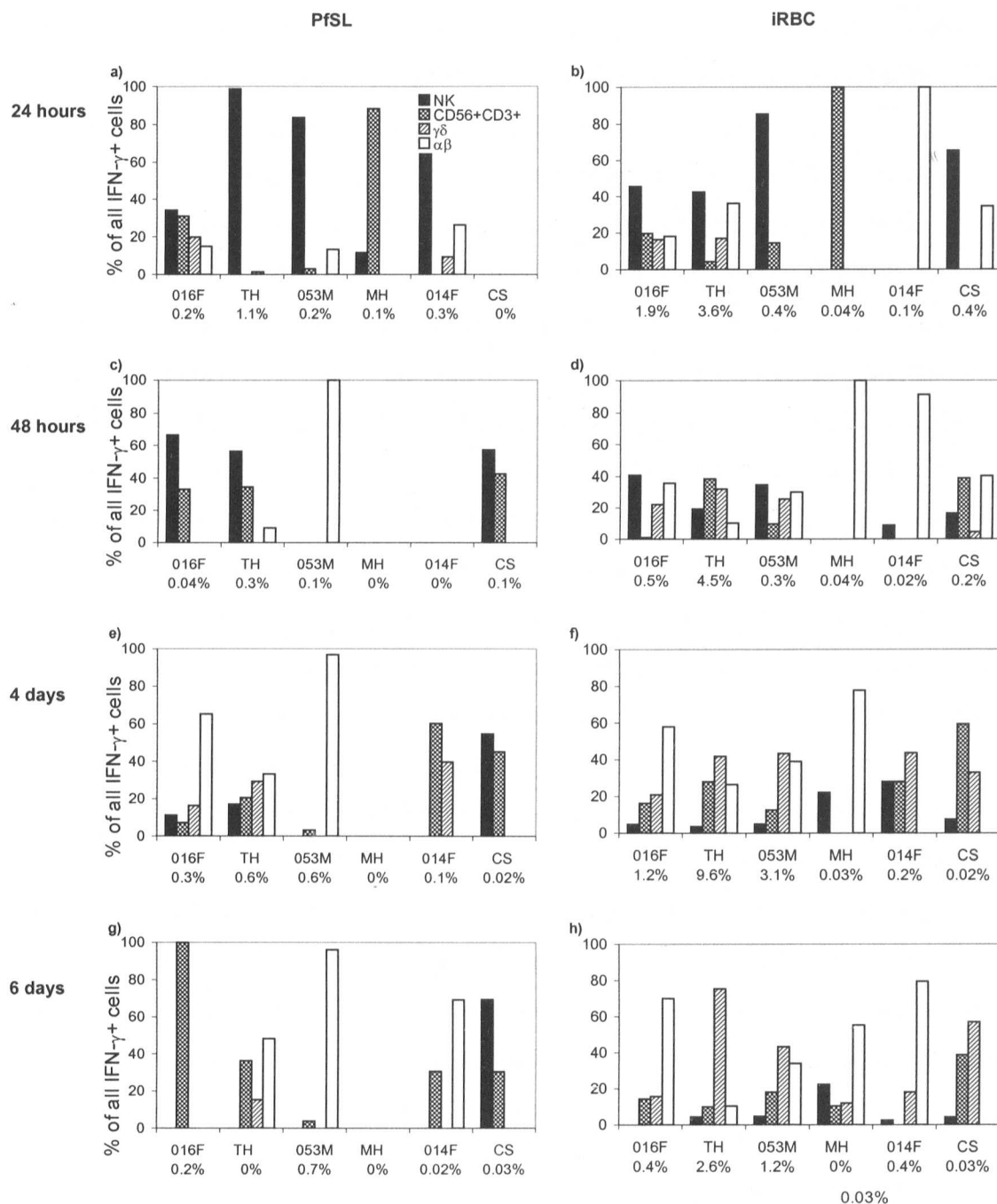


Figure 4.5: Percent of IFN- γ + cells that are $\alpha\beta$, $\gamma\delta$, NK or CD56+CD3+ cells. PBMC (10^6 /ml) from 6 donors were cultured with PfSL, iRBC or uRBC (3×10^6 /ml) for 1, 2, 4 or 6 days. Cells were stained for presence of $\alpha\beta$, $\gamma\delta$ or NK surface phenotypes in combination with intracellular IFN- γ . The percent of all IFN- γ + cells belonging to each surface phenotype are shown for 6 donors at each time-point in response to PfSL (a, c, e, g) or iRBC (b, d, f, h) with uRBC control values already subtracted. Each bar represents a different cell phenotype. The percent of all events that were IFN- γ + are shown underneath the donor names along the x-axes for each time point.

human blood donors (Table 4.2). The number of IFN- γ^+ cells per 100,000 events was calculated for PBMC cultured with uRBC, iRBC or PfSL and the number (%) of the IFN- γ^+ cells that were NK cells (i.e. CD3 $^-$, CD56 $^+$, IFN- γ^+) is also shown.

In both the malaria unexposed and malaria exposed donors there is marked heterogeneity in the number of IFN- γ^+ cells in both PfSL and iRBC cultures. In the malaria unexposed donors, iRBC induced significantly higher numbers of PBMC to produce IFN- γ than did PfSL (Wilcoxon signed rank test, $Z = 2.308$, $p = 0.021$) but this difference was not significant for the malaria exposed donors ($Z = 1.177$, $p = 0.239$). Similarly, in malaria unexposed donors iRBC induced significantly more NK cells (in terms of both absolute numbers and percentages) to produce IFN- γ than did PfSL ($Z = 2.591$, $p = 0.009$) and, again, this difference was not significant for the malaria exposed donors ($Z = 1.255$, $p = 0.209$). The total number of IFN- γ^+ cells, and particularly the number of IFN- γ^+ NK cells, was somewhat lower in the malaria exposed donors than in the malaria unexposed donors, but there was considerable heterogeneity within the malaria exposed group and these differences were not statistically significant (Student's t-test, $t \leq 1.88$, $p \geq 0.07$ for all comparisons).

Perhaps the most noticeable trend in the data was the marked variation between donors in the numbers of IFN- γ^+ NK cells that were induced by iRBC (ranging from 2940 cells per 100,000 events to less than 20). To better illustrate these differences, the percentage of IFN- γ^+ NK cells for each of 18 donors in response to iRBC have been plotted in Figure 4.6a. The heterogeneity is antigen specific and is not due to the inherent inability of a donor's NK cells to produce IFN- γ as proven by the percentage of NK cells making IFN- γ in response to PHA (Figure 4.6b).

	Donor	Ethnicity	Total IFN- γ + cells/100,000			Number (%) of IFN- γ + cells that are NK cells	
			uRBC	PfSL	iRBC	PfSL (%)	iRBC (%)
Malaria unexposed	053M	E	333	2503	4333	1650 (65.9)	2940 (67.9)
	TH	E	1298	1987	4369	568 (28.6)	1736 (39.7)
	094M	As	1318	845	3175	6 (14.1)	1515 (47.7)
	016F	E	125	385	1634	116 (30.1)	1017 (62.2)
	JR	E	841	971	1940	109 (11.2)	934 (48.1)
	095F	E	4329	4944	5179	637 (12.9)	628 (12.1)
	SB	As	673	679	1136	202 (29.7)	561 (49.4)
	073F	E	749	609	1278	79 (13.0)	470 (36.8)
	PC	E	601	1807	1509	221 (12.2)	260 (17.2)
	014F	E	1222	1315	1378	522 (39.7)	240 (17.4)
	CS	E	608	494	900	34 (6.9)	186 (20.7)
	093F	E	760	932	981	55 (5.9)	148 (15.1)
	AJH	E	2256	3037	2361	183 (6.0)	125 (5.3)
	RaT	As	1216	995	1217	70 (7.0)	77 (6.3)
	KAT	E	282	458	282	61 (12.0)	58 (11.7)
	MH	E	632	534	577	56 (10.5)	54 (9.4)
	BdS	As	612	637	612	30 (4.7)	40 (6.5)
	AMSP	Af	474	544	495	5 (1.0)	19 (3.8)
	Mean		1018	1315	1853	256	612
	\pmSE		± 227	± 278	± 344	± 94	± 183
Malaria exposed	JET	E	1245	1963	3343	830 (42.3)	1056 (31.6)
	FMO	Af	556	773	1112	185 (23.9)	537 (48.3)
	HTD5	Af	907	919	1061	118 (12.8)	250 (23.6)
	HTD6	Af	1222	1373	1264	158 (11.5)	149 (11.8)
	HTD3	Af	846	520	1017	45 (8.7)	118 (11.6)
	HT	Af	654	772	807	144 (18.7)	113 (14.0)
	KT	Af	524	615	660	113 (18.4)	103 (15.6)
	HTD1	Af	400	485	348	44 (9.1)	95 (27.3)
	HTD7	E	636	755	1264	84 (1.1)	87 (6.9)
	HTD9	Af	376	356	425	51 (14.3)	67 (15.8)
	HTD8	Af	302	498	291	77 (15.5)	32 (11.0)
	HTD4	Af	540	411	95	30 (7.3)	8 (8.4)
	Mean		684	786	973	156	217
	\pmSE		± 90	± 133	± 243	± 63	± 86

Table 4.2: The early (24 hour) IFN- γ response to *P. falciparum*.

IFN- γ + cells were detected by flow cytometry after 24 hours co-culture of PBMC with malaria infected (iRBC) or uninfected (uRBC) red blood cells or parasite lysate (PfSL). The table shows the total number of IFN- γ + cells per 100,000 events and the number of CD3⁺CD56⁺ IFN- γ + cells. Percentages refer to the proportion of IFN- γ + cells that are NK cells.

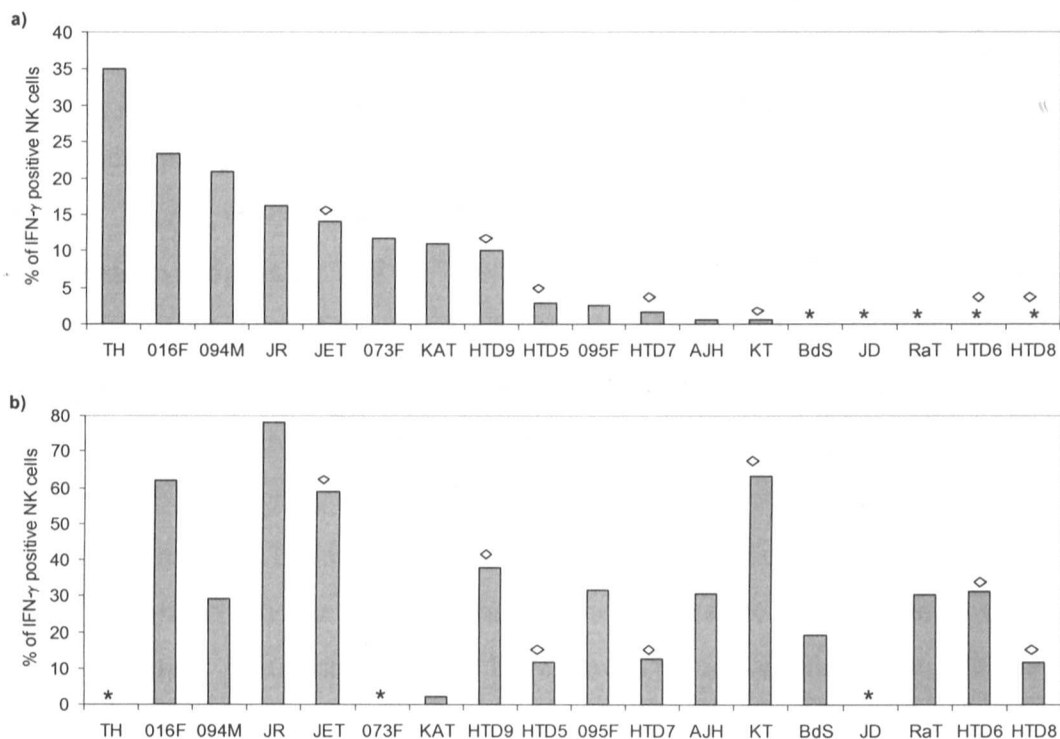


Figure 4.6: Heterogeneity of the human NK cell response to iRBC. The percentage of NK (CD3⁻CD56⁺) cells staining for intracellular IFN- γ after 24 hours co-culture with (a) iRBC (uRBC values subtracted) or (b) PHA (GM values subtracted) is shown. CD56⁺ cells were selected by gating on side scatter and CD56⁺; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportion of CD3-CD56⁺ cells that were positive for IFN- γ is shown; data are based on collection of 100,000 events. Data from 18 individual donors are shown. All donors are malaria naïve except for those marked (◊) who have varying levels of prior malaria exposure. * = no IFN- γ ⁺ NK cells detectable.

4.4 Discussion

It has long been known that T cells from malaria naïve donors can proliferate and secrete cytokines in response to *P. falciparum* antigens. Responses tend to peak after 6 to 7 days activation in vitro and the proliferating cells have been identified as either TCR $\alpha\beta$ ⁺ T cells, which respond in a classical MHC-restricted manner to both live and dead parasite antigens, or TCR $\gamma\delta$ ⁺ T cells that respond preferentially to live parasites (Dick *et al.*, 1996; Waterfall *et al.*, 1998); both cell types have also been shown to secrete IFN- γ (Currier *et al.*, 1992; Goodier *et al.*, 1995; Zevering *et al.*, 1992). Recently, it has been suggested that live *P. falciparum* can induce rapid (within 12 to 24 hours) IFN- γ and TNF- α responses (Scragg *et al.*, 1999) and that TCR $\gamma\delta$ ⁺ T cells may contribute to the early IFN- γ response (Hensmann *et al.*, 2001). The purpose of this set of experiments was, therefore, to conduct a detailed study of the kinetics and cellular sources of IFN- γ production by *P. falciparum*-activated PBMC, with particular emphasis on malaria-naïve donors.

The first interesting observation, gleaned from the ELISA analysis, was that live parasites were much better at stimulating an IFN- γ response than dead parasite lysate. This difference seemed to occur early on and there were differences between donors, with some donors making a rapid and substantial IFN- γ response and others making a modest and delayed response. This was mirrored in the flow cytometric data with large numbers of IFN- γ ⁺ cells in cultures from some donors and very few IFN- γ ⁺ cells in cultures from other donors.

The occurrence of a $\gamma\delta$ T cell-derived IFN- γ response (which came up at the 2-4 day time-point), seemed to correlate with the presence of an early 24-hour NK response. The individuals who didn't mount a $\gamma\delta$ T cell response also seemed to lack NK IFN- γ activity. These data suggest that NK cells may be necessary to "kick-start" the inflammatory response and its subsequent amplification. This hypothesis is supported by experiments by Carnaud *et al.* where they demonstrated that by blocking early IFN- γ production by NKT and NK cells, they were able to delay or entirely eliminate the secondary wave of IFN- γ release (Carnaud *et al.*, 1999).

In the experiments presented here, NKT cells were not fully characterized. In order to be certain that a cell belongs to this lineage, the invariant pairing of the V α 24 and the V β 11 chains of the TCR must be demonstrated. There was a subset of cells with a CD56⁺CD3⁺ phenotype which produced IFN- γ (as seen in Figure 4.3d) but their response peaked at later time-points (4-6 days) and since activated T-cells are known to upregulate CD56 on their surface (Slifka *et al.*, 2000), it is logical to tentatively categorize them as T cells. The kinetics of the $\alpha\beta$ T cell response were much as expected (from Chapter 3), coming up at the later time-points. This echoes previously published data (Dick *et al.*, 1996) (Rhee *et al.*, 2001). The $\gamma\delta$ T cells showed less of a distinct pattern, with the majority of the donors tested having $\gamma\delta$ cells producing IFN- γ at intermediate time-points, with a peak at approximately 4 days. This observation does not mirror results from a study claiming that $\gamma\delta$ T cells are rapidly activated to produce IFN- γ within 18 hours of contact with parasitized erythrocytes (Hensmann *et al.*, 2001). However, the authors do demonstrate that $\gamma\delta$ T cells are more efficiently activated by live rather than dead parasites, an observation that not only confirms the data described in this chapter, but further supports other published data (Waterfall *et al.*, 1998).

When the phenotype of IFN- γ positive cells was analyzed, it was apparent that in individuals who mounted a detectable response to parasites at 24 hours, NK cells represented the largest fraction of activated cells, an observation that was highly reproducible within individual donors (see Chapter 5). The heterogeneity seen among donors could not be explained by differing levels of prior exposure to malaria, although as a group, the malaria-exposed donors did have slightly depressed, albeit not statistically significant, NK-IFN- γ responses as compared to the naïve group.

The contribution of human NK cells to the cytokine response to *P. falciparum* has not been widely addressed, their cytotoxic abilities having been focused on more closely (Ojo-Amaize *et al.*, 1981) (Stach *et al.*, 1986) (Orago *et al.*, 1991). However, in the mouse, the importance of NK-derived IFN- γ has been demonstrated in both the liver stage and the blood stage of infection. The protective immune response of BALB/c mice vaccinated with *P. yoelii* irradiated sporozoites was markedly reduced following anti-asialo GM1 NK cell depletion (Doolan *et al.*, 1999). A similar effect was seen when IL-

12 was neutralized. Combining this data, the authors suggested that CD8 T cell-derived IFN- γ induces IL-12 production which in turn precipitates NK cells to release IFN- γ thereby amplifying the adaptive response (Doolan *et al.*, 1999). Similar results were obtained in *P. yoelii* blood stage infection, where depletion of NK cells resulted in diminished IFN- γ levels followed by an inability to control parasitemia (Choudhury *et al.*, 2000).

Overall, these experiments served to establish NK cells as a significant source of IFN- γ during the first 24-hours of PBMC incubation with live parasites. These NK cells are preferentially activated to produce IFN- γ by live parasites, an observation that has not been previously described to date. However, NK activation by iRBC was not a blanket phenomenon. There was a marked heterogeneity in the level of NK activation (as defined by IFN- γ production) which could not be explained through NK inertia, since donors whose cells didn't respond to iRBC did respond to PHA. These results suggest that NK IFN- γ production is dependent on an additional signal which was absent in certain donors.

Having identified NK cells as a significant source of early IFN- γ in response to live *P. falciparum*, regardless of prior exposure, the mechanism of NK cell activation and parasite recognition needed to be elucidated, a topic which is addressed in the following chapters.

Chapter 5 Activation of NK Cells by *P. falciparum*

5.1 Introduction

In the previous chapter, I have shown that human NK cells can be activated by live, intact parasitized erythrocytes to produce IFN- γ within 24 hours. The data in this chapter explore this phenomenon in more detail, showing the kinetics of the response and the activation requirements for human NK cells.

NK cells can have many functions in infection. They can be activated to kill virus-infected cells through cytotoxic mechanisms or they can be activated to release cytokines. The main cytokine they are able to produce is IFN- γ , and they have been shown to do so in infections by several pathogens including *Leishmania major*, *Mycobacterium tuberculosis* and *Toxoplasma gondii* (Akuffo *et al.*, 1993; Kemp *et al.*, 1997) (Hunter *et al.*, 1994). The mechanisms by which they could be induced to produce IFN- γ can be following direct contact with an infected cell, as is known to happen in viral infections (Arase *et al.*, 2002), a point that will be addressed in the next chapter, or through bystander activation mediated by cytokines (Lertmemongkolkhai *et al.*, 2001). The two main cytokines that have been shown to have a particular role in inducing IFN- γ from NK cells are IL-12 and IL-18.

IL-12 is a heterodimeric cytokine made up of two covalently linked chains, namely p35 which is constitutively expressed and p40 which can be induced. These two chains combine to form the p70 active form of the cytokine which is able to induce the release of IFN- γ (Kobayashi *et al.*, 1989; Trinchieri 1994) which in turn enters into a positive feedback loop to amplify IL-12 production (Kubin *et al.*, 1994). The main sources of IL-12 are macrophages and dendritic cells (D'Andrea *et al.*, 1992; Schoenhaut *et al.*, 1992) which can be stimulated by intracellular as well as extra-cellular pathogens.

The properties of IL-12 were originally described in the mouse by Schoenhaut *et al.* in 1992, where they showed that murine IL-12 was not only able to induce the release of IFN- γ , but was also involved in augmenting NK cytotoxic activity and T-cell

proliferation (Schoenhaut *et al.*, 1992). Subsequent studies illustrated the absolute necessity for IL-12 in the optimal activation of NK cells and their release of IFN- γ , for a wide variety of pathogens both in mice and in humans. In C3H mice infected with *Leishmania major*, antibody neutralization of IL-12 resulted in the in vivo abrogation of NK-derived IFN- γ and NK-mediated cytotoxicity (Scharton-Kersten *et al.*, 1995). Similarly, *Listeria monocytogenes* and *Toxoplasma gondii* infections in SCID mice were found to stimulate macrophage release of IL-12 and subsequent production of IFN- γ from NK cells (Gazzinelli *et al.*, 1993; Tripp *et al.*, 1994; Tripp *et al.*, 1993). Finally, IL-12 was found to be crucial for NK activation in viral infections in both mice and humans (Orange *et al.*, 1996; Orange *et al.*, 1994).

The importance of IL-12 in NK stimulation and IFN- γ induction has been demonstrated for malaria as well. By administering controlled doses of exogenous IL-12 to *P. chabaudi* AS susceptible mice, Stevenson *et al.* were able to increase resistance and prolong survival, which they correlated with high levels of splenic and circulating IFN- γ (Stevenson *et al.*, 1995). In a subsequent study, the authors identified NK cells as the key mediators in IFN- γ induced protection, showing that these cells responded most efficiently following IL-12 administration (Mohan *et al.*, 1997). IL-12p40 knock-out mice, which lack functional IL-12, have a reduced early IFN- γ response (and subsequent antibody response) when challenged with *P. chabaudi* AS. This weakened immune response resulted in higher blood-stage parasitemia, more severe anemia and decreased survival time (Su *et al.*, 2002).

IL-12 is not the only cytokine implicated in inducing IFN- γ ; IL-12 has been shown to synergize with other cytokines, particularly IL-18 which was first described in 1989 and named IFN- γ inducing factor (IGIF) after it's major observed effect (Nakamura *et al.*, 1989). IL-18 is produced by monocyte/macrophages in both mice and humans (Ushio *et al.*, 1996) in a precursor form which is inactive until processed by caspase 1 (Gu *et al.*, 1997). In addition to being able to stimulate TNF- α production (Puren *et al.*, 1998) IL-18 has the ability to augment FAS ligand-mediated cytotoxicity in NK cells (Tsutsui *et al.*, 1996) as well as to stimulate NK cells to release IFN- γ (Garcia *et al.*, 1999).

The role of IL-18 in protection from murine malaria has been investigated using BALB/c mice and the lethal *P. berghei* ANKA parasite. It was found that antibody-mediated neutralization of IL-18 resulted in a more severe disease course encompassing higher parasitemia, and shorter survival. The investigators hypothesized that the function of IL-18 in this system is to optimize IFN- γ release and to promote Th1-like cellular immunity (Singh *et al.*, 2002).

Although both IL-18 and IL-12 are able to induce the release of IFN- γ from murine NK cells, optimal activation occurs when both these cytokines are present and are able to synergize (Okamura *et al.*, 1995). Furthermore, individual addition of either of these cytokines to human NK cells in vitro failed to induce IFN- γ (Fehniger *et al.*, 1999), whereas simultaneous addition of both IL-12 and IL-18 resulted in the release of copious amounts of IFN- γ (Fehniger *et al.*, 1999; Micallef *et al.*, 1996). Similarly, the combined addition of IL-12 and IL-18 to murine NK cells resulted in the release of high levels of IFN- γ (Tomura *et al.*, 1998), and IL-18/IL-12 double knock-out mice displayed a more severe defect in NK cell function than their single IL-18 knock-out counterparts which, in turn, displayed a reduced NK-derived IFN- γ production phenotype (Takeda *et al.*, 1998). Therefore, the combined release of IL-12 and IL-18 activates NK cells to release IFN- γ , which may in turn stimulate macrophages to release, for example, NO and O₂⁻ (Okamura *et al.*, 1998). The synergism of IL-12 and IL-18 in malaria infection has not been investigated, however one can hypothesize that it is important given the established anti-parasitic functions of IFN- γ (Chizzolini *et al.*, 1990) as well as NO and its derivatives (Rockett *et al.*, 1991).

In the following series of experiments I have attempted to characterize the minimal requirements for human NK cells to produce IFN- γ in response to *P. falciparum* stimulation. In particular, I have analyzed the relative importance of IL-12 and IL-18 in NK cell activation and IFN- γ production in response to *P. falciparum*.

5.2 Materials and Methods

PBMC separation, cell culture conditions, FACS staining, antigen preparations and IFN- γ and IL-12 ELISA are described in Chapter 2.

5.2.1 Cytokine titration

IL-2, IL-12 and IL-18 (all Peprotech, London, UK) were added in combination to PBMC cultures of 3 donors (RaT, RhD and FMO) to assess the amounts needed to elicit a strong IFN- γ response from NK cells. The range of concentrations tested were based on the manufacturer's ED₅₀ values for each of the three cytokines (0.1-0.5 ng/ml for IL-2, 0.1-0.2 ng/ml for IL-12 and 5-10 ng/ml for IL-18).

5.2.2 IL-12 and IL-18 neutralization

PBMC were cultured at 1×10^6 /ml with 3×10^6 iRBC or uRBC for 24 hours in the presence of neutralizing antibody to human IL-12 or IL-18 or both neutralizing antibodies together. Neutralizing goat anti-human IL-12 polyclonal antibody (R&D Systems) was added to PBMC cultures at concentrations ranging from 0.5 to 10 μ g/ml. An isotype matched control antibody (goat IgG, Sigma) was used at the same concentrations. Neutralizing mouse polyclonal anti-human IL-18 or a mouse IgG1 control antibody (both R&D Systems) were used at concentrations from 0.1 to 5.0 μ g/ml. Cells were stained as stated in Chapter 2 and NK IFN- γ release was quantified using flow cytometric analysis.

5.2.3 IL-12 and IL-18 addition

PBMC were cultured at 1×10^6 /ml with 3×10^6 iRBC, PfSL or uRBC for 24 hours in the presence of increasing concentrations of recombinant human IL-12 or IL-18 or IL-12 and IL-18 together (Peprotech) (0.1 ng/ml to 10 ng/ml). Cells were then stained and analyzed by FACS to assess the effect of each cytokine on the NK-derived IFN- γ .

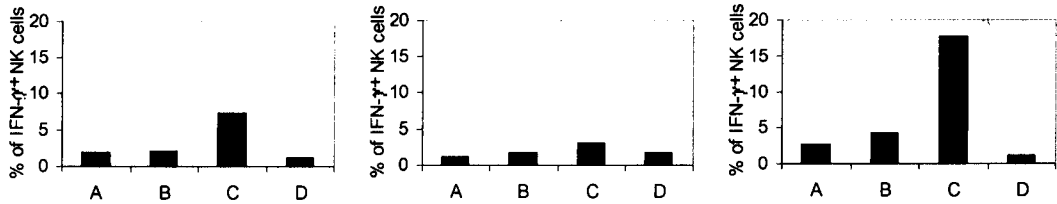
5.3 Results

5.3.1 Selection of a positive control for NK cell activation

Since IL-18 and IL-12 have been shown to act synergistically to induce IFN- γ induction from human NK cells (Fehniger *et al.*, 1999; Micallef *et al.*, 1996), it was deduced that a combination of these cytokines would serve as an acceptable positive control for the detection of NK-derived IFN- γ by flow cytometry. Several attempts to induce an IFN- γ response from these cells with IL-12 and IL-18 at various concentrations failed to do so consistently (data not shown). This finding does not contradict data from Fehniger *et al.* since they used IL-18 at 100 ng/ml in combination with 10 ng/ml of IL-12. Using IL-18 at such a high concentration was not practical within the realm of this study. Since IL-2 is commonly used to make NK cells proliferate (and hence become activated) IL-2 was added to the cytokine cocktail, as shown in Figure 5.1 for three different donors. Using the company-derived ED₅₀ values (Peprotech) for each of the three cytokines as the starting point, three different combinations of cytokines were used, with combination C (IL-2 at 2 ng/ml, IL-12 at 0.4 ng/ml, IL-18 at 20 ng/ml) proving to be the most potent. However, as illustrated in Figure 5.1a, this combination did not yield consistent results across different donors and was therefore not satisfactory as a positive control. This result was not due to defective reagents, since all three recombinant cytokines were biologically active as demonstrated in Figure 5.1b. Increasing amounts of each cytokine were added to PHA (1 μ g/ml) activated PBMC and the IFN- γ release from NK cells was quantified by FACS. There is a dose-dependent increase in the NK cell-IFN- γ response for all three cytokines.

A previous study at LSHTM [Kazi (PhD thesis, LSHTM 2002)] had determined that in order to get more than 50% of NK cells staining positive for IFN- γ , one needed to add in the order of 100 ng/ml of each cytokine. Seeing as this was an impractically large amount to use repeatedly, I tried using a sub-optimal amount of PHA (1 μ g/ml) to induce NK IFN- γ production, presumably through bystander activation via CD3 cells (Figure 5.2). PHA consistently induced IFN- γ in NK cells from all donors tested. Although the

a)



b)

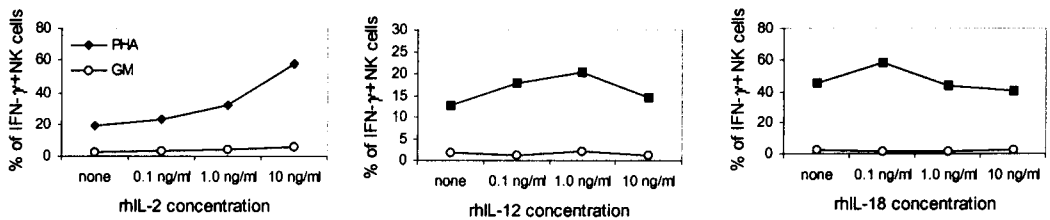


Figure 5.1: Induction of IFN- γ from NK cells by varying concentrations of IL-2, IL-12 and IL-18.

(a) PBMC (10^6 /ml) from three different donors were cultured with increasing amounts of IL-2, IL-12 and IL-18 in combination for 24 hours. PBMC were subsequently stained for flow cytometric analysis. CD56⁺ cells were selected by gating on side scatter and CD56⁺; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportions of CD3⁺CD56⁺ cells that were positive for IFN- γ are shown; data are based on collection of 100,000 events.

A= IL-2 0.5 ng/ml, IL-12 0.2 ng/ml, IL-18 10 ng/ml

B= IL-2 1 ng/ml, IL-12 0.4 ng/ml, IL-18 20 ng/ml

C= IL-2 2 ng/ml, IL-12 0.4 ng/ml, IL-18 20 ng/ml

D= unstimulated negative control.

(b) To confirm that all three recombinant cytokines were biologically active, PHA stimulated (1 μ g/ml) or unstimulated PBMC (10^6 /ml) from an additional three donors were cultured with increasing amounts of each cytokine. Cells were analyzed by FACS as described above.

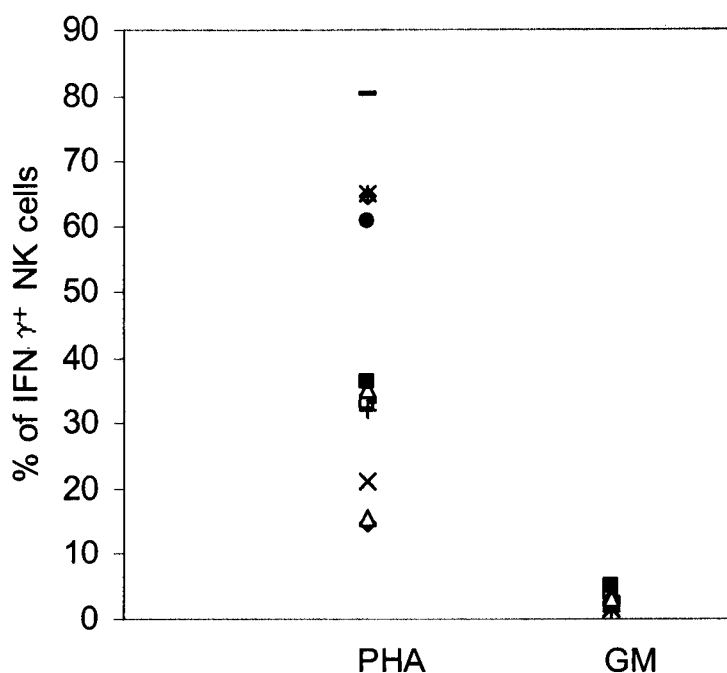


Figure 5.2: PHA as a positive control for NK cell activation.

PBMC ($10^6/\text{ml}$) from 12 different donors (each represented by a different symbol) were cultured with PHA ($1\text{ }\mu\text{g/ml}$) for 24 hours, with GM serving as the unstimulated, negative control. CD56⁺ cells were selected by gating on side scatter and CD56⁺; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportion of CD3⁺CD56⁺ cells that were positive for IFN- γ is shown; data are based on collection of 100,000 events.

magnitude of the response varied, the effect of PHA was highly significant ($t=6.0777$, $df=11$, $p<0.0001$).

5.3.2 Selection of an optimal malaria antigen concentration

Previous studies from our lab indicate that 1×10^5 schizonts per million cells is the optimal concentration ratio for T cell ($\alpha\beta$ and $\gamma\delta$) activation for a 6-day period (Dick *et al.*, 1996). Other studies which focused on a shorter time course used 1×10^6 schizonts per million cells (Hensmann *et al.*, 2001) (Scragg *et al.*, 1999). Therefore, I performed a titration incubating PBMC with 1×10^5 , 1×10^6 or 1×10^7 iRBC, PfSL or uRBC for 24 hours, staining for NK markers and intracellular IFN- γ and analyzed the cells by flow cytometry to determine the percentage of NK cells activated to produce IFN- γ . The data for two donors are shown in Figure 5.3. As is apparent, 1×10^5 is not a high enough concentration to induce a response, whereas 1×10^6 is effective and 1×10^7 is best of all. I decided to use the intermediate value of 3×10^6 schizonts/ 1×10^6 PBMC since a ratio of 3 parasitized RBC per PBMC is a physiological parasite concentration, equating to an iRBC count of 15,000 parasites per microliter of blood in an infected person (symptomatic malaria patients typically have parasite counts of between 10,000 and 200,000 parasites per microliter).

5.3.3 Kinetics of IFN- γ production from NK cells

In order to identify the optimal time-point to detect cytokine production by NK cells a time-course analysis was carried out for IFN- γ production. TNF- α production was also analyzed since NK cells are known to produce both of these cytokines (Mendes *et al.*, 2000) in response to other pathogens and since they are both important in anti-malarial effector mechanisms and in malaria pathology. PBMC were incubated with PHA, PfSL, iRBC, uRBC for periods from 3 hours up to 3 days and TNF- α (figure 5.4a) or IFN- γ (figure 5.4b) production by NK cells was assessed at each of 6 time-points. Although up to 20% of NK cells stained for TNF- α after incubation with PHA for 15 hours, TNF- α production in response to malaria antigen was minimal and barely above background. IFN- γ production was much more pronounced with up to 60% of NK cells

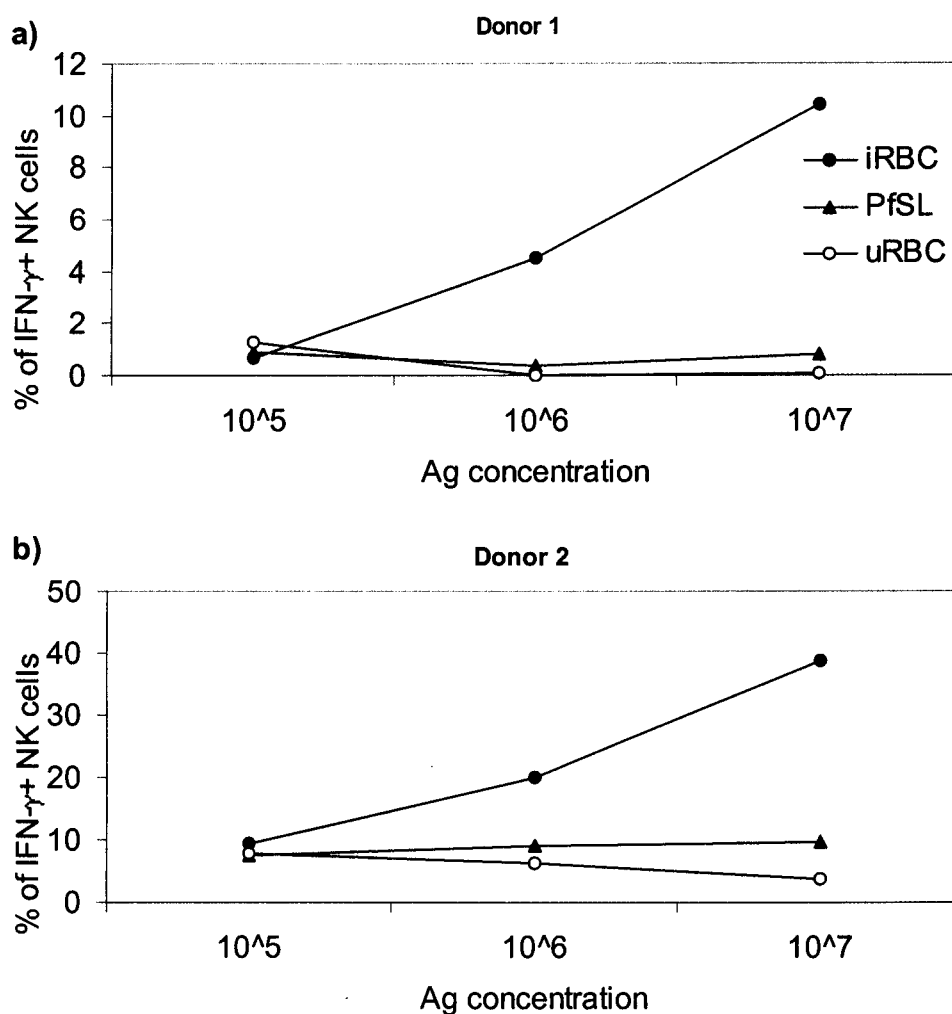


Figure 5.3: Parasite titration. PBMC ($10^6/\text{ml}$) were cultured with iRBC, PfSL or uRBC at increasing concentrations for 24 hours. PBMC were subsequently stained for flow cytometric analysis. $\text{CD}56^+$ cells were selected by gating on side scatter and $\text{CD}56^+$; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportions of $\text{CD}3^+ \text{CD}56^+$ cells that were positive for IFN- γ are shown for two donors (a,b); data are based on collection of 100,000 events.

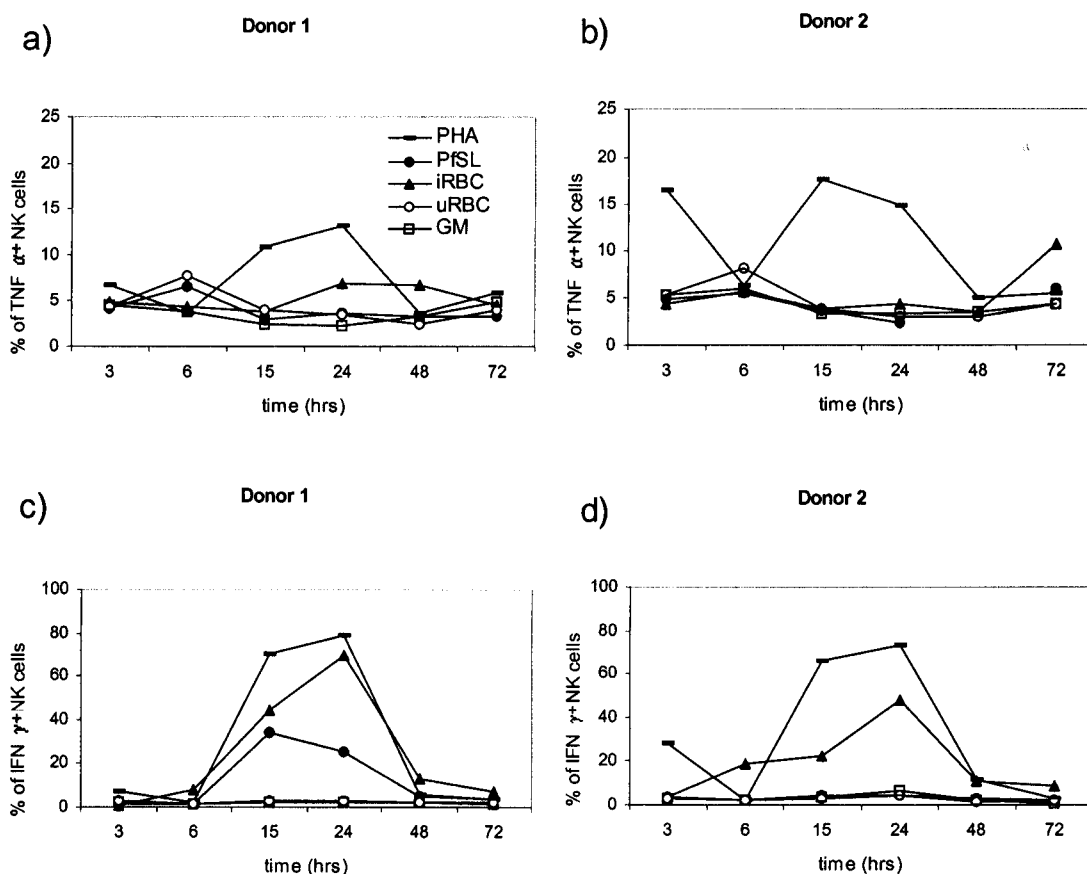


Figure 5.4: Kinetics of cytokine production by CD56⁺CD3⁻ NK cells.

The kinetics of the TNF-α (a,b) or IFN-γ (c,d) response were determined for two donors by culturing PBMC (10⁶/ml) with iRBC, PfSL, uRBC (3 x 10⁶/ml) or PHA (1 μg/ml) for up to 72 hours. PBMC were subsequently stained for flow cytometric analysis. CD56⁺ cells were selected by gating on side scatter and CD56⁺; the gated cells were then analyzed for intracellular cytokine and CD3 expression. The proportions of CD3⁻CD56⁺ cells that were positive for TNF-α (a,b) and for IFN-γ (c,d) are shown; data are based on collection of 100,000 events. Note the differences in scale on the vertical axes.

staining for IFN- γ after iRBC stimulation; the IFN- γ response peaked at 15-24 hours, sharply decreasing thereafter. Therefore, an incubation time of 24 hours was adopted for all further experiments involving NK-derived IFN- γ detection.

5.3.4 Stability of individual NK responses to malaria

The data in Figures 5.3, 5.4 and Chapter 4, show some variation between donors in the magnitude of the NK cell response. In order to determine whether this was due to stable, reproducible differences between donors, I investigated the NK response of individual donors over time. The consistency of the NK iRBC-induced IFN- γ response over time for individual donors was assessed using data from four separate experiments conducted over the course of a year. Figure 5.5 illustrates the percentage of iRBC-induced IFN- γ ⁺ NK cells in a high responder 053M (figure 5.5a) and for a low responder MH (figure 5.5b). Although there is about a 5% fluctuation between experiments, it is plainly apparent that 053M is consistently a high responder and that MH is consistently low. The overall percentage of PBMC that were of the NK phenotype was also relatively consistent over time for both the high and the low responders (Figure 5.5c).

5.3.5 IL-12 and IL-18 neutralization

As activation of NK cells is known to be at least partially IL-12 and IL-18 dependent in many systems (Fehniger *et al.*, 1999) (Lertmemongkolchai *et al.*, 2001) (Orange *et al.*, 1996), it was hypothesized that the difference between donors in their ability to make an NK cell response to malaria might be due to differences in their ability to make IL-12 or IL-18 in response to iRBC activation. To determine whether NK cell responses to malaria were indeed IL-12 or IL-18 dependent, I incubated PBMC with iRBC or uRBC for 24 hours in the presence or absence of increasing concentrations of neutralizing antibody to human IL-12 or IL-18 (Figure 5.6). The percentage of IFN- γ ⁺ NK cells was markedly reduced (by approximately 50%) in the presence of 0.5 μ g/ml anti-human IL-12, but not by equivalent concentrations of an isotype-matched antibody (Figure 5.6a); NK cell responses were not further reduced by increasing doses of antibody. In a similar titration experiment, anti-IL-18 antibody inhibited NK activation by up to 50% at high concentrations (5.0 μ g/ml) (Figure 5.6b).

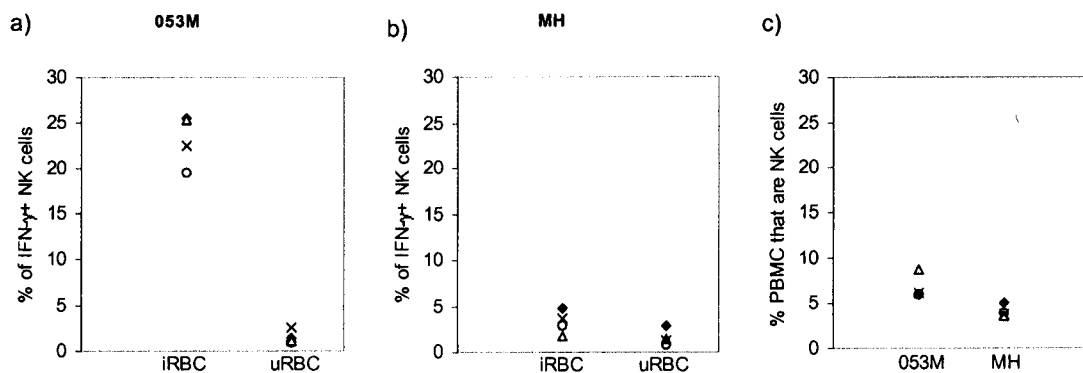
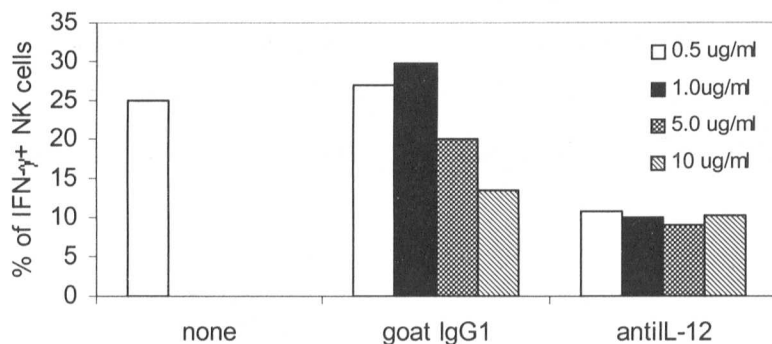


Figure 5.5: Reproducibility of the NK-IFN- γ response in individual donors.

PBMC (10^6 per ml) from (a) a high responder (053M) and (b) a low responder (MH) were cultured with 3×10^6 iRBC, or 3×10^6 uRBC for 24 hours on four separate occasions, each indicated by a different symbol. CD56 $^{+}$ cells were selected by gating on side scatter and CD56 $^{+}$; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportion of CD3 $^{-}$ CD56 $^{+}$ cells that were positive for IFN- γ is shown; data are based on collection of 100,000 events. The percentage of all PBMC that were NK cells (CD56 $^{+}$ CD3 $^{-}$) are shown in (c) for each bleeding.

a)



b)

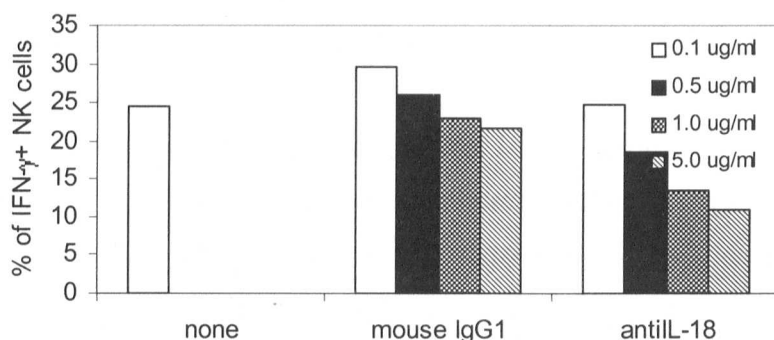


Figure 5.6: Anti-IL-12 and anti-IL-18 antibody titrations. PBMC's from a single donor (10^6 per ml) were cultured with 3×10^6 iRBC, or 3×10^6 uRBC for 24 hours in the presence of increasing amounts of neutralizing antibody to human IL-12 (a) or IL-18 (b) or control, isotype matched antibodies. Control cultures were incubated with 3×10^6 uRBC; uRBC values have been subtracted from iRBC values. CD56⁺ cells were selected by gating on side scatter and CD56⁺; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportion of CD3⁺CD56⁺ cells that were positive for IFN- γ is shown; data are based on collection of 100,000 events.

Using anti-IL-12 at 0.5 µg/ml and anti-IL-18 at 5.0 µg/ml, 5 donors were screened to see if this effect was consistent. Anti-IL-12 consistently reduced the percentage of IFN- γ ⁺ NK cells by between 50 and 100% (Figure 5.7a), whereas anti-IL-18 was able to partially inhibit induction of IFN- γ in NK cells of some donors (e.g. 094M and FMO) but not others (053M) (Figure 5.7b). Neutralizing both cytokines together resulted in a pattern of inhibition of NK-derived IFN- γ production similar to that if anti-IL-12 alone (Figure 5.7c).

5.3.6 Supplementation of IL-12 and IL-18

As the NK cell response to iRBC was clearly IL-12-dependent, I wondered whether the difference between high and low responder donors was due to differences in IL-12 induction from macrophages or dendritic cells in the PBMC culture. An attempt was made to measure IL-12 in culture supernatants by ELISA, but levels were too low to be detected.

As I could not directly measure IL-12 (or IL-18) production, I considered whether adding additional IL-12 and/or IL-18 to the cultures would a) allow non-responding donors to make an NK IFN- γ response to *P. falciparum* and b) augment the NK response to schizont lysate (PfSL) to the levels seen for iRBC (live parasitized erythrocytes). Addition of rIL-12 at concentrations from 0.1 to 10.0 ng/ml to PBMC cultured with either PfSL or iRBC had no consistent effect on either the concentration of IFN- γ in supernatants (Figure 5.8a) or the percentage of IFN- γ ⁺ NK cells after 24 hours (Figure 5.8b). Importantly, addition of exogenous rIL-12 did not enhance the response of low responding donors and did not enhance PfSL-induced responses to the level of iRBC-induced responses. Thus, although IL-12 plays an essential role in the activation of NK cells to make IFN- γ , lack of IL-12 is insufficient to explain the differences in IFN- γ responses between individuals or between responses induced by live and dead parasites.

In a comparable experiment, addition of rIL-18 to 24 hour PBMC cultures with PfSL or iRBC had no effect on the overall IFN- γ release or the NK cell response to PfSL as measured by ELISA and FACS, respectively (Figure 5.9a and 5.9b). However, low

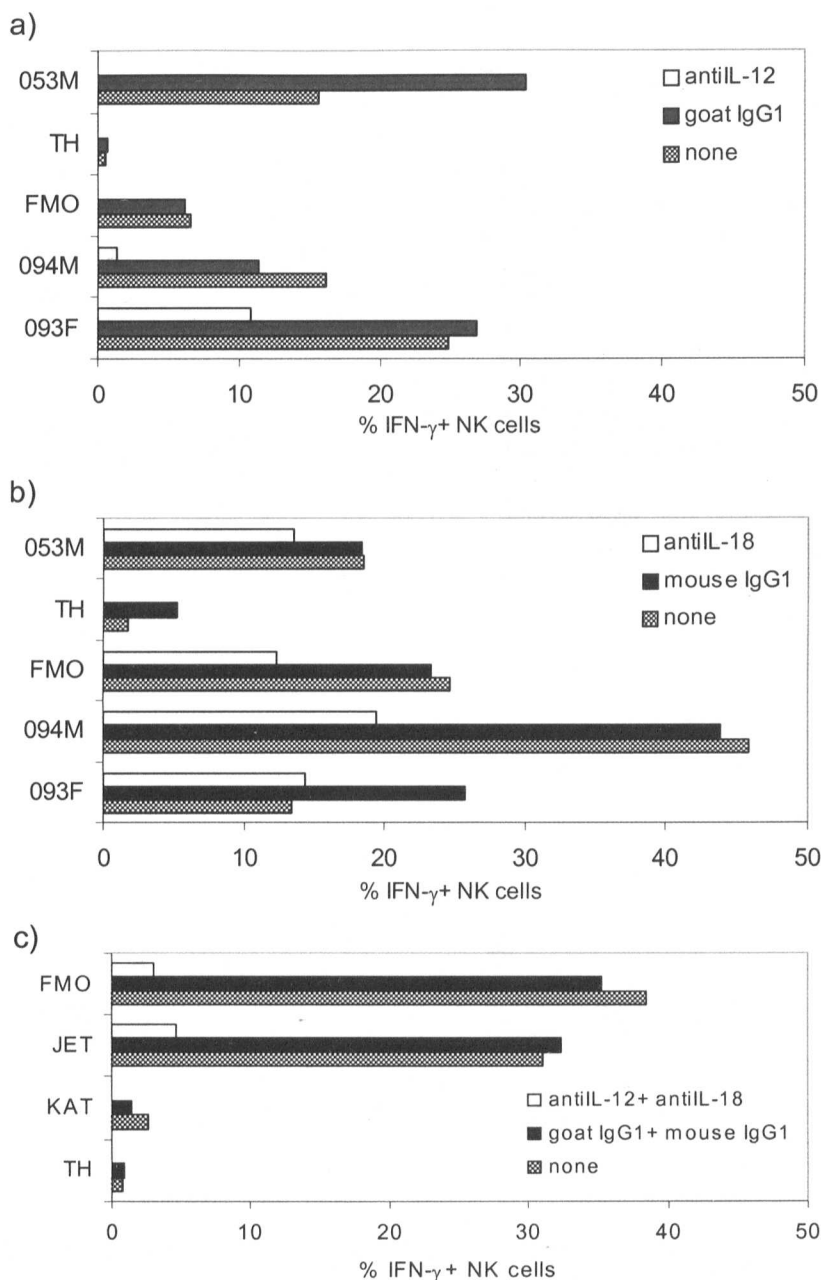


Figure 5.7: Effect of neutralizing IL-12 and IL-18 on NK-derived IFN- γ production. PBMC's (10^6 per ml) were cultured with 3×10^6 iRBC, or 3×10^6 uRBC for 24 hours in the presence of neutralizing antibody to human IL-12 (a) or IL-18 (b) or IL-12 and IL18 together (c) or control, isotype matched antibodies. uRBC values have been subtracted from iRBC values. CD56 $^+$ cells were selected by gating on side scatter and CD56 $^+$; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportion of CD3 $^-$ CD56 $^+$ cells that were positive for IFN- γ is shown; data are based on collection of 100,000 events.

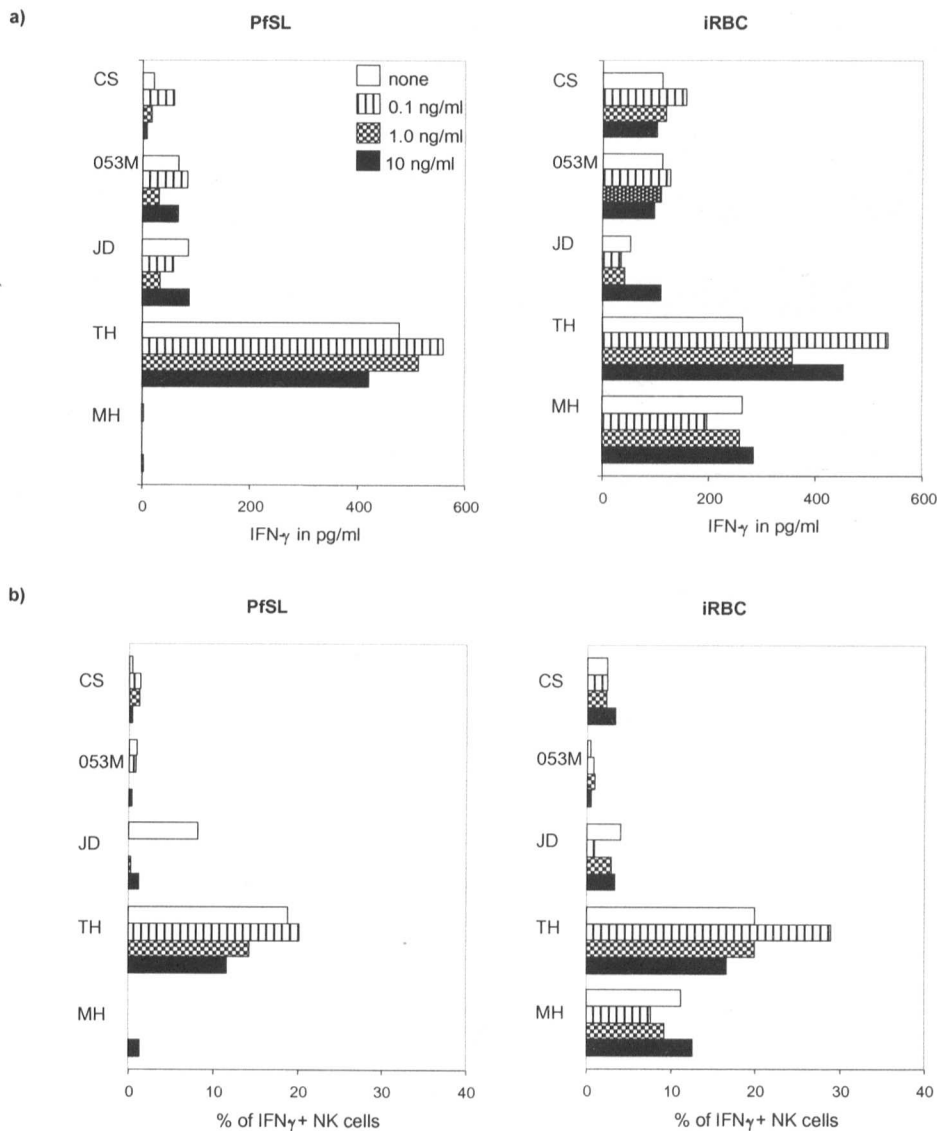


Figure 5.8: Effect of exogenous rIL-12 on NK-derived IFN- γ .

PBMC's (10^6 per ml) were cultured with 3×10^6 iRBC or 3×10^6 freeze-thawed schizonts (PfSL) for 24 hours in the presence of increasing concentrations of rhIL-12. Control cells were cultured with 3×10^6 uninfected red blood cells (uRBC); uRBC values have been subtracted from iRBC and PfSL values. IFN- γ production was assessed by ELISA (a) and by the proportion of NK cells that are IFN- γ^+ (b). CD56 $^+$ cells were selected by gating on side scatter and CD56 $^+$; the gated cells were then analyzed for IFN- γ and CD3 expression. Data are based on collection of 100,000 events.

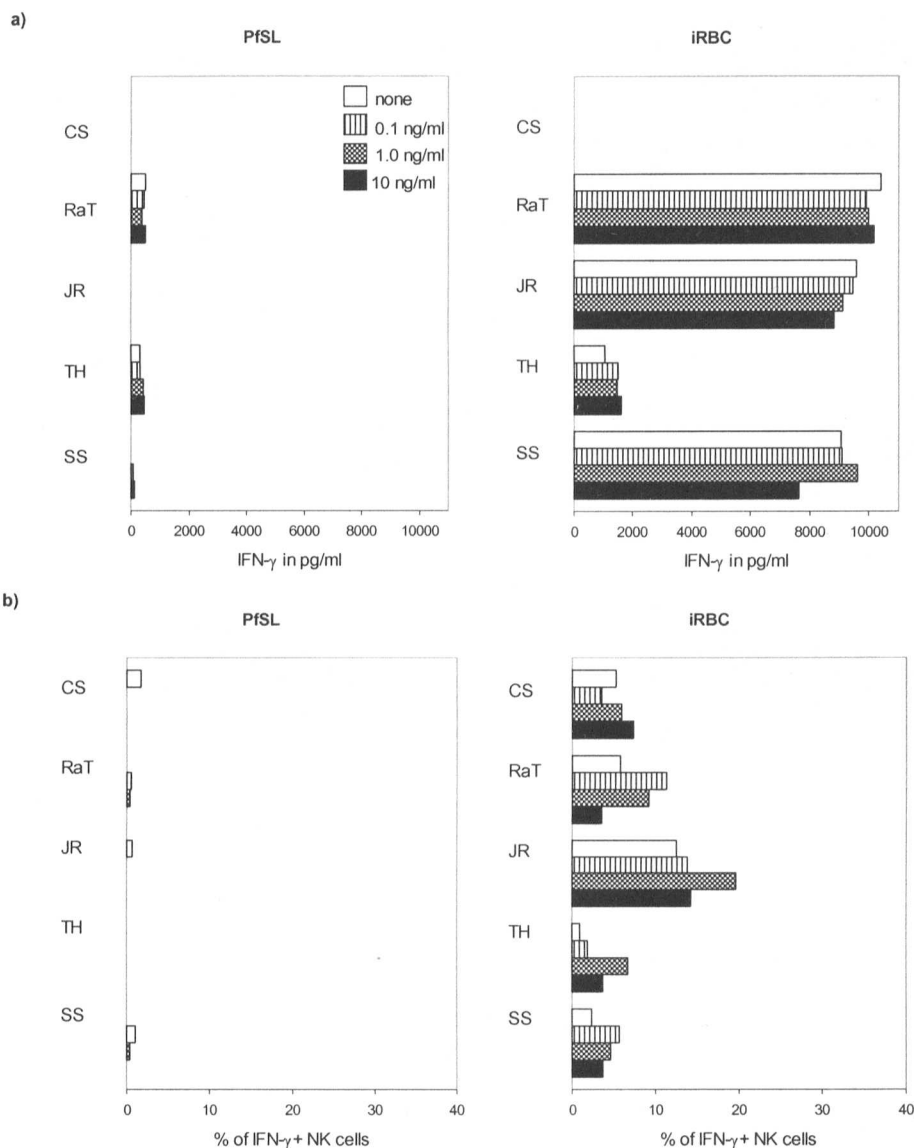


Figure 5.9: Effect of exogenous rIL-18 on NK-derived IFN- γ .

PBMC's (10^6 per ml) were cultured with 3×10^6 iRBC or 3×10^6 freeze-thawed schizonts (PfSL) for 24 hours in the presence of increasing concentrations of rhIL-18. Control cells were cultured with 3×10^6 uninfected red blood cells (uRBC); uRBC values have been subtracted from iRBC and PfSL values. IFN- γ production was assessed by ELISA (a) and by the proportion of NK cells that are IFN- γ^+ (b). CD56 $^+$ cells were selected by gating on side scatter and CD56 $^+$; the gated cells were then analyzed for IFN- γ and CD3 expression. Data are based on collection of 100,000 events.

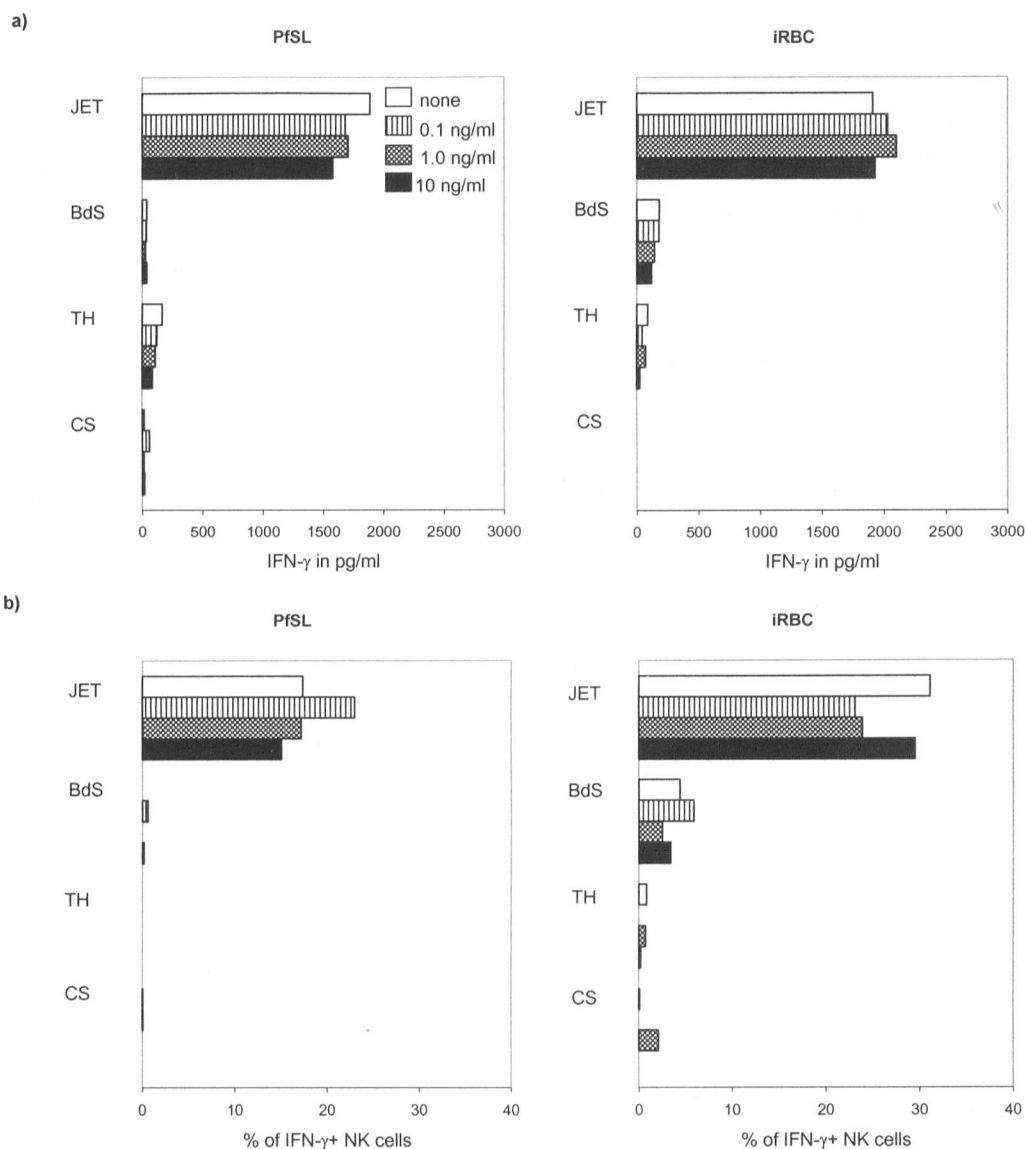


Figure 5.10: Effect of exogenous rIL-12 and rIL-18 together on NK-derived IFN- γ . PBMC's (10^6 per ml) were cultured with 3×10^6 iRBC or 3×10^6 freeze-thawed schizonts (PfSL) for 24 hours in the presence of increasing concentrations of rhIL-12 and rhIL-18 from 0.1 to 10 ng/ml of each cytokine. Control cells were cultured with 3×10^6 uninfected red blood cells (uRBC); uRBC values have been subtracted from iRBC and PfSL values. IFN- γ production was assessed by ELISA (a) and by the proportion of NK cells that are IFN- γ^+ (b). CD56 $^+$ cells were selected by gating on side scatter and CD56 $^+$; the gated cells were then analyzed for IFN- γ and CD3 expression. Data are based on collection of 100,000 events.

doses of rIL-18 (0.1 or 1.0 ng/ml) did marginally enhance the NK response to iRBC in most donors (Figure 5.9b). Adding both cytokines together at concentrations ranging from 0.1 to 10 ng/ml, also had a minimal effect on NK IFN- γ responses (Figure 5.10a and 5.10b).

5.4 Discussion

In this series of experiments the kinetics of NK-derived IFN- γ release and some of the mechanisms through which NK cells are activated during *in vitro* stimulation with *P. falciparum* were explored. Although NK-derived IFN- γ can be detected as early as 6 hours, its production seems to peak at between 15 and 24 hours and to disappear by three days. A related observation, which echoes the overall IFN- γ kinetics described in Chapter 4, was that live parasites induced a strong IFN- γ response from NK cells whereas dead parasites did not. Differences between live and dead parasites have been noticed previously, particularly in their ability to activate TCR $\gamma\delta^+$ T cells (Waterfall *et al.*, 1998) (Hensmann *et al.*, 2001). This observation has now been extended to include preferential induction of IFN- γ production in NK cells by live parasitized erythrocytes. The requirement for live parasites within intact red blood cells for induction of the innate response may be due to a need for direct contact between the parasitized red cell and the leukocyte (be it a lymphocyte or an accessory cell) or may be due to instability of the parasite-derived ligands that interact with cell surface receptors. These parasite-derived ligands may interact directly with NK cells or may mediate their effect via accessory cells (such as macrophages and dendritic cells) in the PBMC culture. The NK response to live parasites, although consistent over time for cells derived from a single donor, showed marked variation between donors, leading to the identification of individuals as high or low responders.

As both IL-12 and IL-18 have been shown to be required for optimal NK cell activation (Fehniger *et al.*, 1999) the possibility that differences between donors in the magnitude of the IFN- γ response might be due to differences in IL-12 or IL-18 induction was considered. It was not surprising to find that the NK cell response to malaria was highly IL-12 dependent as this is widely reported for NK cells (Fehniger *et al.*, 1999), and has been shown for NK responses to a number of pathogens (Orange *et al.*, 1996) (Lertmemongkolchai *et al.*, 2001). However, addition of rIL-12 did not significantly enhance the response of individual donors and did not increase the response to PfSL to

levels seen for iRBC, suggesting that lack of IL-12 production in the cultures was not sufficient to explain the low NK responses of some donors or the low response to parasite lysate.

The requirement for IL-18 was less clear, with some donors showing a reduction in NK cell responses to iRBC in the presence of anti-IL-18 antibody and cells from other donors being unaffected. On the other hand, low doses of rIL-18 did marginally enhance the proportion of NK cells that could be induced to produce IFN- γ in response to iRBC in most donors indicating that IL-18 can indeed augment the NK response to malaria parasites. Taking the IL-12 and IL-18 data together, it appears that whilst monokines – produced for example by dendritic cells or macrophages in the PBMC cultures – are required to optimize the NK cell response to *P. falciparum*, these monokines are not sufficient on their own to induce the levels of IFN- γ production obtained with malaria-infected red blood cells. A lack of IL-12 or IL-18 in the culture medium is insufficient to explain the very low levels of NK cell activation induced by parasite lysates or the very low responses to iRBC in some donors. However, the influence of other cytokines (e.g. IL-2 and IL-15), and the ability of an individuals' macrophages or dendritic cells to respond to malaria antigens in other ways, requires further investigation.

Having ruled out differences in IL-12 and IL-18 as the source of individual variation, we considered that diversity in expression of NK cell receptors, which might interact with parasite-derived ligands, might explain our findings. This hypothesis is investigated in the next chapter.

Chapter 6 Are Direct Interactions Between NK Cells and *P. falciparum* Required for Optimal NK Cell Activation?

6.1 Introduction

The purpose of the experiments outlined in this chapter was to determine if direct contact between NK cells and parasitized erythrocytes occurs and, if so, whether such contact is necessary for optimal activation of these cells, as defined by IFN- γ production. Published data has correlated NK lytic activity with parasitemia, showing that NK cell levels in peripheral blood of children with *P. falciparum* infection were raised above normal and that their cytotoxic abilities seemed simultaneously enhanced (Orago *et al.*, 1991). Likewise, a study comparing immune and non-immune adults, showed that incubating PBMC with *P. falciparum* schizont lysate enhanced their lytic activity against the NK-sensitive cell line K562 (Theander *et al.*, 1987). Although these studies do not prove that this cytotoxicity is directed against the parasite itself, they do show that malaria infection has an activating effect on the NK cell population, whether it be a direct effect, or a bystander effect mediated by cytokines released from other cell types.

However, there is evidence that NK cells can directly recognize the parasite in that, in a chromium release assay, NK cells derived from Kenyan adults and children with or without acute *P. falciparum* infection were able to lyse parasitized red blood cells (Orago *et al.*, 1991). The authors revealed that NK cells, regardless of donor group, can lyse parasitized erythrocytes and that this process involves protein kinase C, since the addition of neomycin interfered with cytolysis of the targets. These data imply direct recognition of parasitized erythrocytes by NK cells, which begs the question of how this might occur.

NK cells characteristically recognize target cells through ligation of MHC Class I, the level of which triggers an activating or inhibitory signal (Moretta *et al.*, 2001). Since erythrocytes do not express MHC on their surface, the assumption is that the interaction is mediated through an, as of yet, unidentified autologous ligand or alternatively, that a *P. falciparum*-derived ligand is involved. To date, the only pathogen-derived ligand known

to be recognized by NK cells is the cytomegalovirus (CMV) MHC homologue UL18 gene product (m144 and m157 in MCMV). The molecules are expressed as decoys, binding to inhibitory NK cell receptors, and thereby shielding the infected cell from NK lysis, as proved by work using a recombinant MCMV with a disrupted m144 gene which was shown to have severely impaired replicating abilities as compared to the wild type (Farrell *et al.*, 1997) More recently, the ligand for the m157 homologue was identified as the murine Ly49H activating receptor which, once bound, conferred resistance to MCMV infection (Arase *et al.*, 2002) (Smith *et al.*, 2002). Conversely, in MCMV susceptible mice, the m157 molecule was bound by an inhibitory receptor, ultimately leading to disease (Arase *et al.*, 2002).

In this chapter, I attempt to further characterize the need for, and the nature of, the direct contact between NK cells and parasitized erythrocytes, and how inhibiting this contact affects IFN- γ production by these cells.

6.2 Materials and Methods

PBMC separation, cell culture conditions, FACS staining and antigen preparations were done as described in Chapter 2.

6.2.1 Conjugation of erythrocytes and NK cells

To determine if NK cells and parasitized erythrocytes can form stable conjugates, PBMC at 1×10^6 cells/ml were incubated with 3×10^6 iRBC/ml or an equal concentration of live uRBC for a maximum of 3 hours. Cell mixtures were briefly centrifuged (1 minute at 500 g) to bring cells into contact. Cultures were sampled every 30 minutes to assess the progression of conjugate formation. Cells were fixed in 4% paraformaldehyde (PFA) to retain cell attachment, erythrocytes were stained with PE-labeled anti-human glycophorin A antibody (Pharmingen) in conjunction with anti-CD56-FITC and anti-CD3-Tricolor for NK cells, and analyzed by FACS as described in Chapter 2.

6.2.2 Transwell System

In order to assess the need for iRBC-NK contact for the induction of NK-derived IFN- γ release, a transwell system was set up where cells were separated from parasitized erythrocytes but were still exposed to soluble antigenic components and monokines produced by macrophages and dendritic cells in the PBMC culture. Figure 6.1 illustrates the setup.

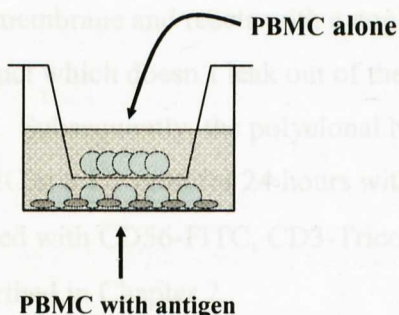


Figure 6.1: Transwell setup.
Illustration of PBMC and parasite antigen incubated in a transwell system.

PBMC at 1×10^6 cells/ml were placed in a tissue culture well with 3×10^6 iRBC, uRBC, or $1 \mu\text{g/ml}$ of PHA. Additional PBMC at an equal concentration were then placed in a

transwell with a 0.4 μm porous polycarbonate membrane (Corning Inc, Bucks, UK) and lowered onto the culture well so that all cells were submerged in culture medium, but the cells within the upper well were not in contact with iRBC or uRBC. Since erythrocytes are approximately 8 μm in diameter and merozoites are approximately 1 μm in diameter, the membrane did not allow traffic of solid antigen but did allow soluble parasite products and cytokines to flow freely between both cell preparations. Plates were then cultured for 24 hours and PBMC were subsequently harvested, and stained with anti-CD3, anti-CD56 and anti-IFN- γ fluorescent antibodies before being analyzed by FACS, as previously described in Chapter 2, to determine the level of NK cell IFN- γ production.

6.2.3 IFN- γ production by NK cell lines and clones

NK cell lines and clones were prepared by K. Eleme (Imperial College, London) according to the protocol described in Chapter 2. Cell lines and clones were incubated with iRBC or uRBC at the previously determined 1:3 ratio (i.e. 10^6 cells/ 3×10^6 iRBC) and then stained with CD56-FITC, CD3-Tricolor and IFN- γ -PE and analyzed by FACS as described in Chapter 2.

6.2.4 Mixing polyclonal NK cell population with PBMC

Several NK clones were mixed together to achieve an oligoclonal population. These cells were stained with 0.5 μM Cell Tracker Green (Molecular Probes, Eugene, Oregon USA) at 37°C for 1 hour and then in 10% autologous plasma to absorb any unbound reagent. Cell Tracker Green is a fluorescent probe which passes through the cell membrane and reacts with cytoplasmic thiol groups, to produce an impermeable product which doesn't leak out of the cell. It can retain its fluorescence at 37°C for 3 days. Subsequently, the polyclonal NK cell mixture was incubated with autologous PBMC at a 1:1 ratio for 24 hours with iRBC or uRBC at a 1:3 ratio. Cells were then stained with CD56-FITC, CD3-Tricolor and IFN- γ -PE and analyzed by FACS as described in Chapter 2.

6.3 Results

6.3.1 NK cells can form stable conjugates with parasitized erythrocytes

In order to determine if NK cells can directly bind to parasitized erythrocytes, PBMC were incubated with iRBC or live uRBC for 30 minute intervals up to a maximum of 3 hours. Cultures were fixed and then stained with anti-CD56, anti-CD3 and anti-glycophorin A. Since glycophorin A identifies erythrocytes, the assumption was that an NK cell (CD56+ CD3-) staining positive for glycophorin A would indicate the attachment of a red blood cell. Therefore, the analysis was done by gating on all CD56+ cells and then seeing how many were negative for CD3 (i.e. NK cells) and positive for glycophorin A (i.e. had bound RBC) (Figure 6.2). The kinetics of attachment were investigated for several donors (3 of which are shown in Figure 6.3) by calculating the percent of all NK cells that were conjugated to erythrocytes at each time-point, and it was determined that a 90 minute incubation was an optimal time for detection of conjugates. Cells from additional donors were subsequently tested at 90 minutes using the same protocol (Figure 6.4). Although there was notable heterogeneity among donors in how efficiently their NK cells formed conjugates, the results were nevertheless highly significant (Wilcoxon signed rank test, $z=2.66$, $n=9$, $p=0.007$).

6.3.2 Direct contact with iRBC is needed for maximum NK-derived IFN- γ production

In order to determine if the attachment of the NK cell to the parasitized erythrocyte plays a role in the induction of IFN- γ from these cells, or if they produce this cytokine solely as a result of bystander activation by monokines, PBMC were incubated using a transwell system. This setup allowed for the simultaneous assaying of NK cells exposed to all parasite-derived antigens including intact infected RBC, and NK cells only exposed to soluble antigenic components and monokines. Cells from six donors were tested (Figure 6.5); cells from both the inner (iRBC free) and the outer (iRBC rich) wells were harvested and stained for the presence of CD56 and IFN- γ and the absence of CD3.

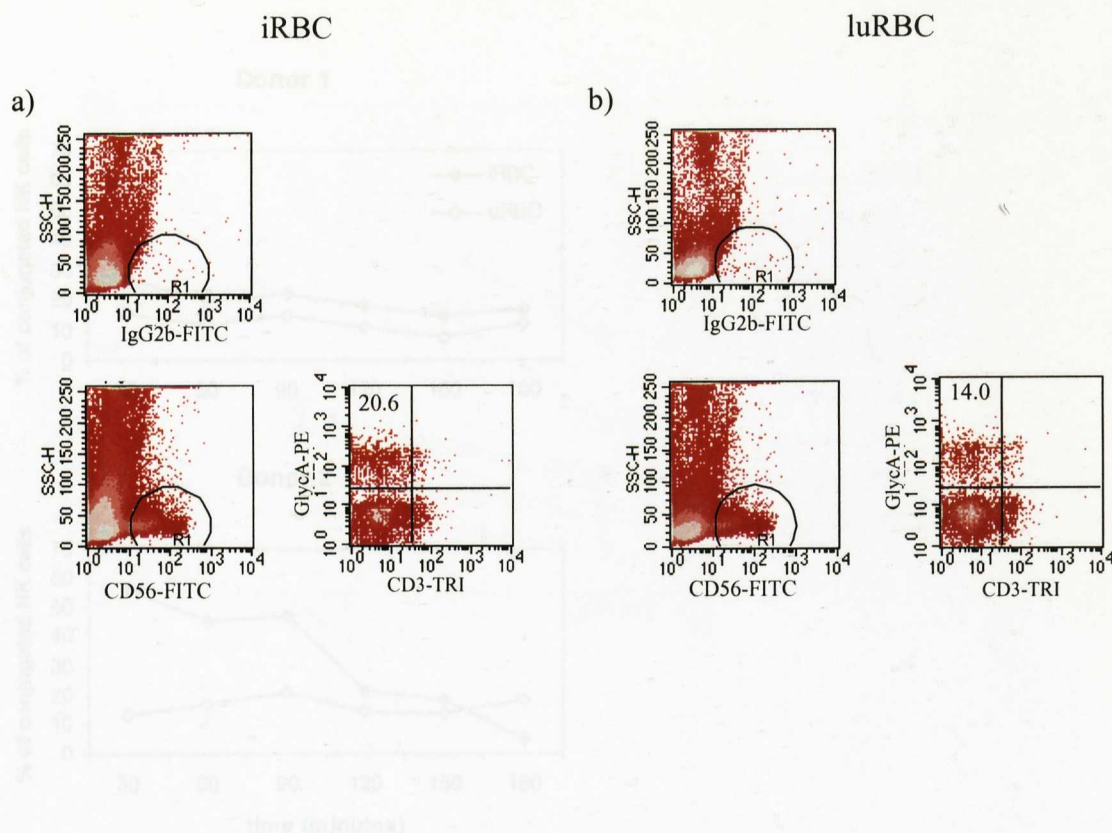


Figure 6.2: Example of conjugate analysis. PBMC's (10^6 per ml) were cultured with 3×10^6 live, parasitized red blood cells (iRBC) (a) or 3×10^6 live, uninfected red blood cells (luRBC) (b), for 30 minute intervals up to a maximum of 3 hours. In each trio, the upper plot shows the isotype control data, the bottom left plot shows the CD56+ cells, and the bottom right plot shows the percent of CD3-/glycophorin A+ cells. Cell surface phenotypes were analyzed by flow cytometry. CD56+ cells were gated and analyzed for CD3 and glycophorin A expression. A total of 100,000 events was counted.

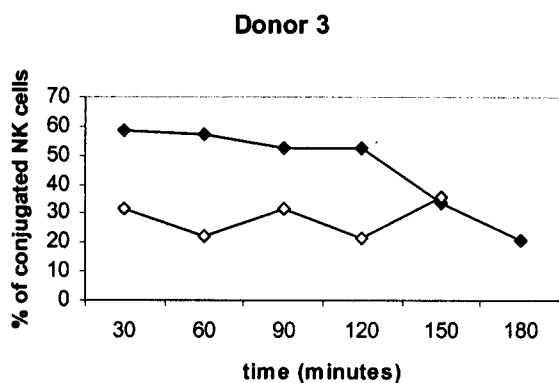
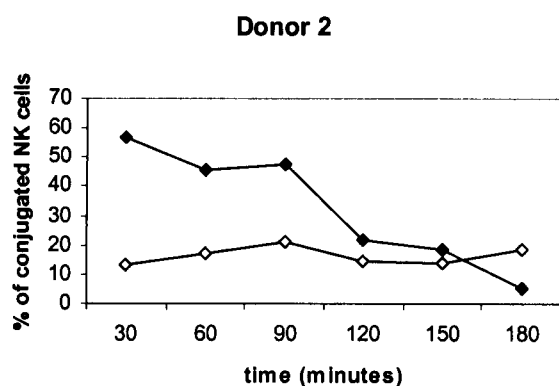
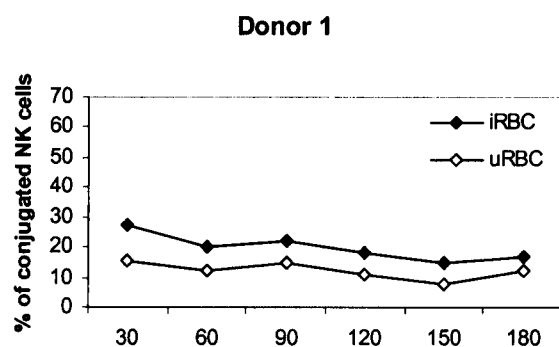


Figure 6.3: Kinetics of NK cell conjugate formation with iRBC or uRBC.

The percentage of NK cells staining positive for glycophorin A, thereby indicating the formation of a conjugate with a red blood cell, was calculated for 3 donors every 30 minutes up to a maximum of 3 hours. Values for parasitized and unparasitized, live erythrocytes are shown. [Value for 180 minutes for Donor 3, not available]

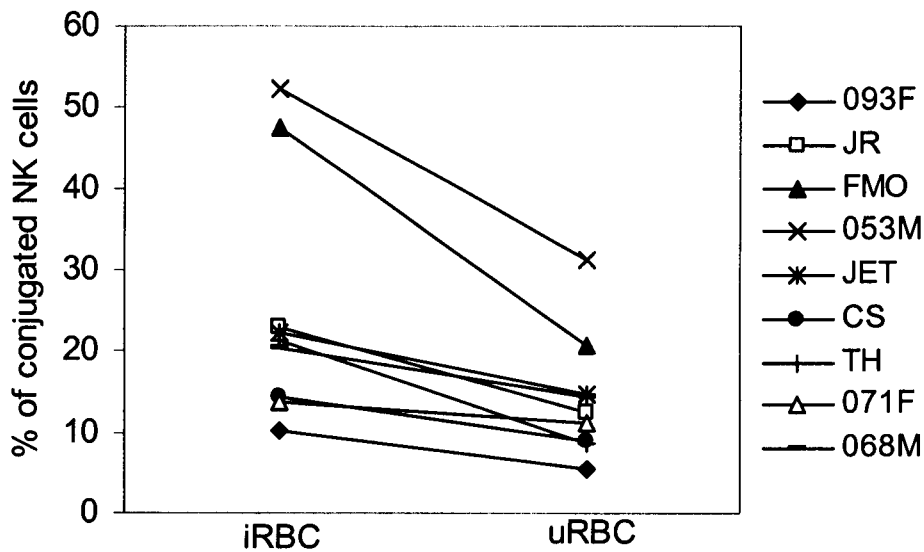


Figure 6.4: NK cell conjugate formation with parasitized or unparasitized erythrocytes.

The percentage of NK cells staining positive for glycophorin A, indicating the formation of a conjugate, was calculated for 7 donors after a 90 minute incubation time. Values for parasitized and unparasitized, live erythrocytes are shown.

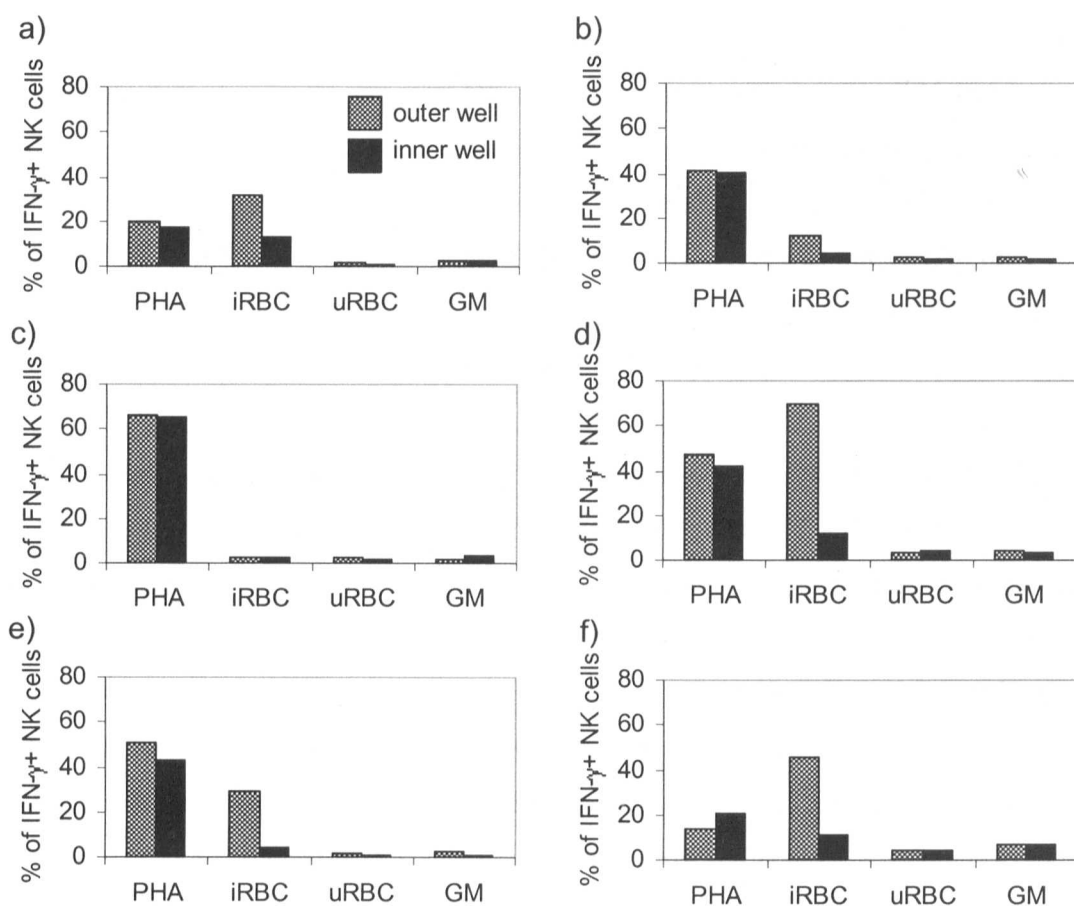


Figure 6.5: Contact between iRBC and NK cells is required for optimal IFN- γ induction.

PBMC from 6 different donors at 1×10^6 cells/ml were incubated for 24 hours with or without direct contact with 3×10^6 iRBC or uRBC as illustrated in Figure 6.1. PHA ($1 \mu\text{g/ml}$) and GM (growth medium, unstimulated) were used as a positive and negative control, respectively. Cells in both the tissue culture well and the transwell were harvested and analyzed for intracellular IFN- γ production and NK cell surface phenotype (CD56+CD3-) as previously described in Figure 4.2. A total of 100,000 events was collected.

PHA was used as a positive control for both the inner and outer wells since it is a soluble antigen, and uRBC and GM (unstimulated) were used as negative controls. In 5 out of the 6 donors tested, the percentage of NK cells staining for intracellular IFN- γ was significantly reduced in the iRBC free wells as compared to the corresponding outer well (Wilcoxon signed rank test, $z=2.2$, $n=6$, $p=0.03$). In one case where no difference was discerned the donor was a non-responder to iRBC. This effect was not seen in the PHA stimulated wells where the percent of responding NK cells was roughly equal in outer and inner wells (Wilcoxon signed rank test, $z=1.2$, $n=6$, $p=0.2$).

6.3.3 Contact on its own is insufficient for stimulation of IFN- γ from NK cells

In order to determine if contact between the NK cell and the iRBC is sufficient for the induction of IFN- γ , purified NK cell lines and clones were prepared from previously determined high responding donors, and these cells were then incubated with parasitized erythrocytes in exactly the same manner as for PBMC. Cells were tested for purity (i.e. lack of CD3+ cells) before being used. Following the 24 hour incubation, cells were stained for NK markers and IFN- γ before being analyzed by FACS. Since this set of experiments involved purified NK cell populations, only 10,000 events were collected. An example of the analysis performed is illustrated in Figure 6.6. All clones or cell lines were gated on a forward and side scatter and subsequently analyzed for CD56 and IFN- γ expression following stimulation with iRBC (a and c) or uRBC (b and d). The CD56+ cells seemed to separate into CD56 bright and CD56 dim population, with most of the IFN- γ coming from the brighter cells.

Three clones from each of two donors (Figure 6.7a), and the corresponding cell lines (Figure 6.7b), were tested. Although there seemed to be a slight trend of an increased percent of IFN- γ + clones after incubation with iRBC compared with uRBC, the difference was too small to be deemed significant, with less than 1% of cells staining positive for IFN- γ in all cases (note scale of y-axis). The same held true for the cell lines. Since I had previously shown that IL-12, and to a lesser extent IL-18, were essential for optimal NK cell activation, I hypothesized that the absence of IL-12 and IL-18-producing cells in the purified NK cell cultures prevented their activation.

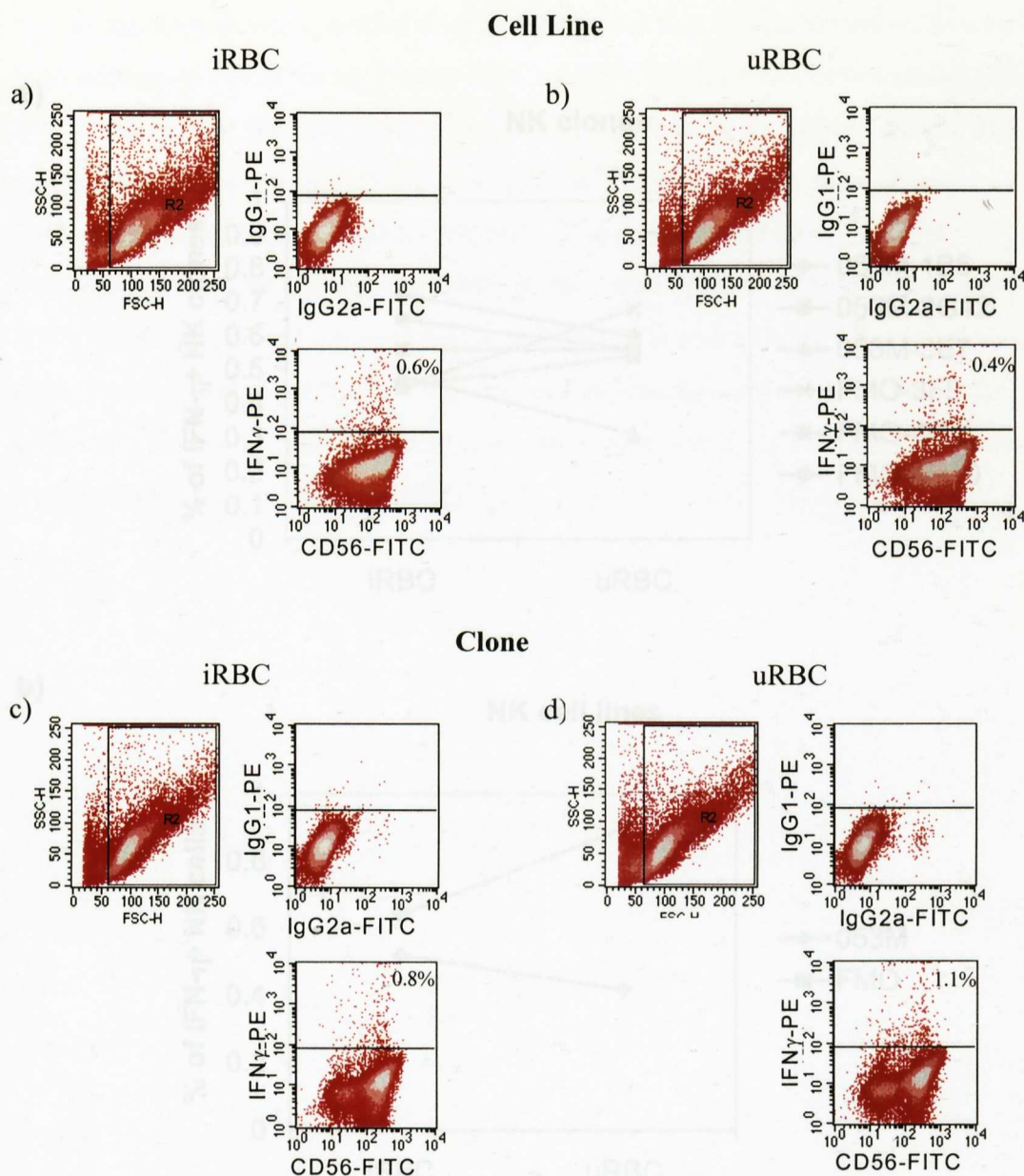
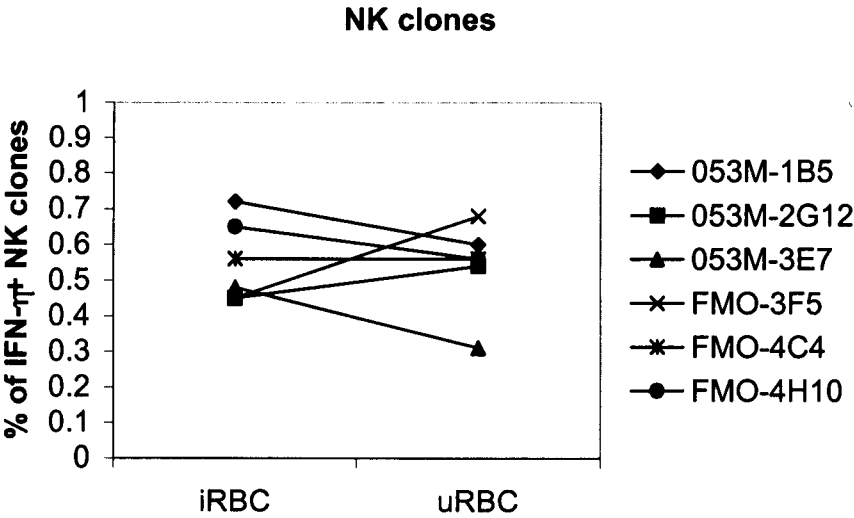


Figure 6.6: NK cell line and clone analysis.

NK cell lines and clones were tested for purity preceding their use, and any containing cells which stained positive for CD3 were discarded. Cells were incubated at a 1:3 ratio with iRBC or uRBC for 24 hours and then analyzed for intracellular IFN- γ . An example of the cell line analysis is shown in (a) and (b) and an example of the clone analysis in (c) and (d). Numbers in the upper right quadrants represent the percent of all gated cells falling in that quadrant. In each set of plots, the upper left one is FSC/SSC and the lower right is the CD56 versus IFN- γ staining. The upper right plot is the corresponding isotype control. A total of 10,000 events was collected.

a)



b)

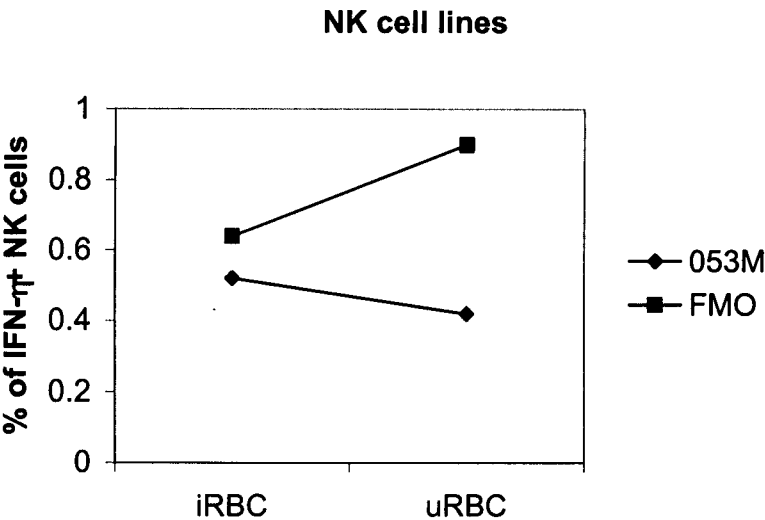


Figure 6.7: NK cell line and clone IFN- γ production in response to iRBC. Cells were incubated at a 1:3 ratio with iRBC or uRBC for 24 hours and then analyzed for CD56 expression and intracellular IFN- γ . All live cells were gated on SSC-FSC and then analyzed to see what percentage were producing IFN- γ . A total of 10,000 events was collected. Results for 3 NK clones from each of two donors (a) and the corresponding NK cell lines (b) are illustrated for the iRBC stimulus and the uRBC negative control.

To test this hypothesis, a purified oligoclonal NK cell line was incubated on its own or with autologous PBMC in addition to iRBC or uRBC for 24 hours before staining for IFN- γ . In parallel, PBMC were also assayed on their own as a positive control. The purified NK cells were stained with Cell TrackerTM Green in order to be able to differentiate them from the PBMC-NK cells. The results obtained support the hypothesis, in that NK clones mixed with autologous PBMC were now able to produce IFN- γ (Figure 6.8e) at levels comparable to PBMC NK cells (Figure 6.8a), whereas on their own they were not (Figure 6.8c). The corresponding analysis is shown for the uRBC (Figure 6.8 b, d and f). This experiment was repeated for 3 donors and the same trend was observed in each case (Figure 6.9).

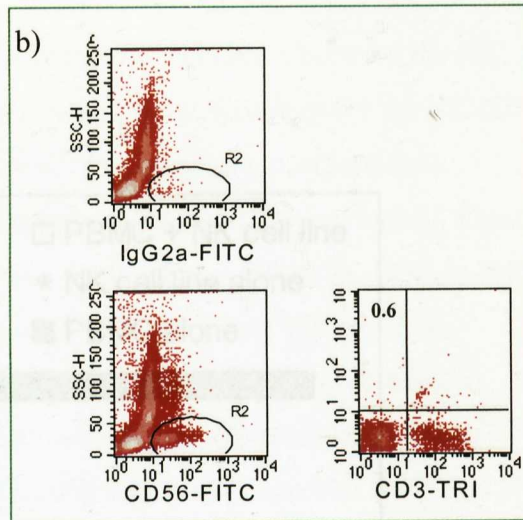
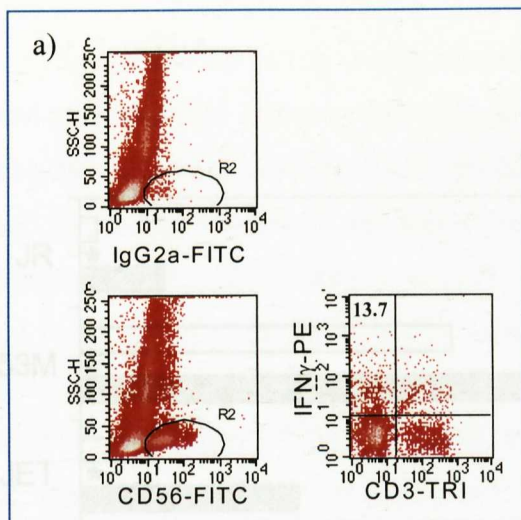
Figure 6.8: IFN- γ production of NK cell clones incubated with or without autologous PBMC-example of analysis.

NK clones were mixed together to achieve an oligoclonal population which was stained with Cell Tracker Green and then incubated for 24 hours with or without autologous PBMC in addition to iRBC or uRBC in a 1:3 ratio. PBMC alone were cultured in parallel to ascertain the donor's NK response with iRBC (a) or uRBC (b). CD56⁺ cells were gated and then examined for CD3 and IFN- γ expression. Isotype controls are also shown. The percentage in the upper left quadrant represents the percent of NK cells producing IFN- γ . Cell lines were cultured alone with iRBC (c) or uRBC (d), or with an equal number of PBMC and iRBC (e) or uRBC (f). Cell lines were gated on the basis of cell tracker green staining and analyzed for IFN- γ production. Numbers in the upper right quadrants represent the percent of cloned NK cells positive for IFN- γ . A total of 10,000 cloned cells were collected.

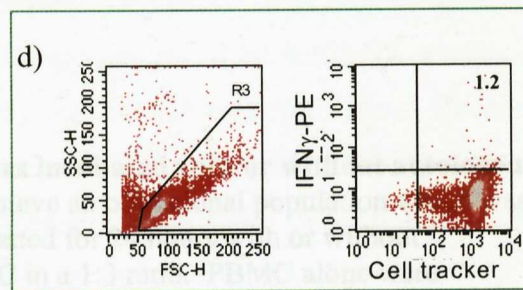
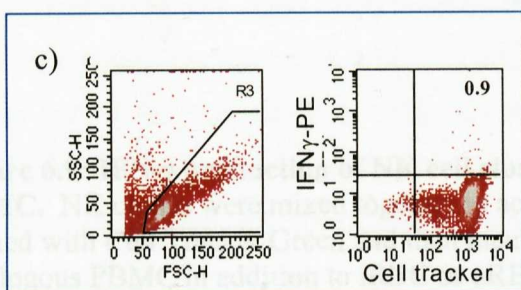
iRBC

PBMC

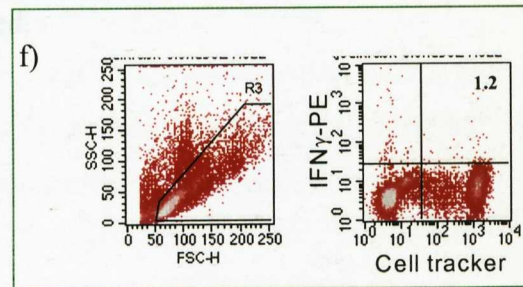
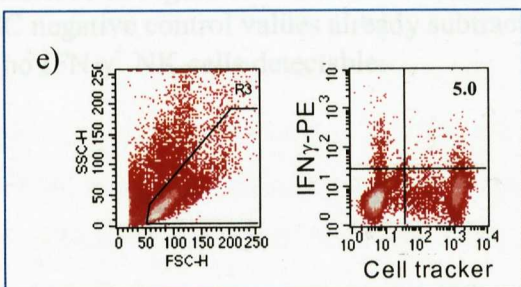
uRBC



Clones



PBMC + Clones



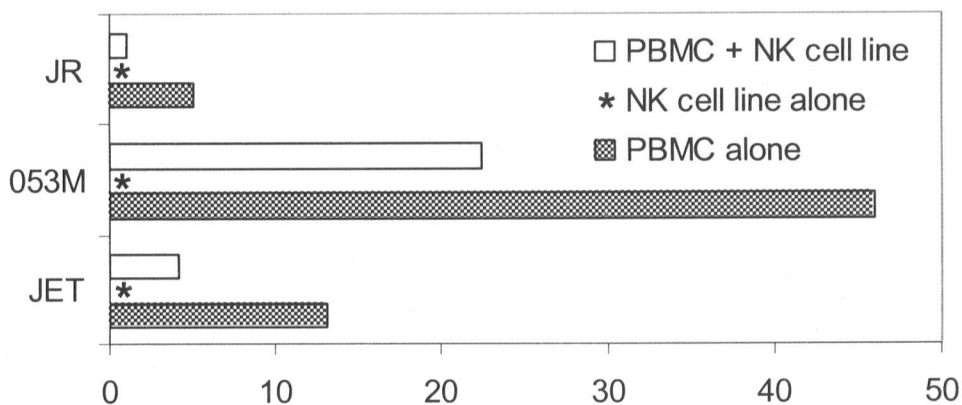


Figure 6.9: IFN- γ production of NK cell clones incubated with or without autologous PBMC. NK clones were mixed together to achieve an oligoclonal population which was stained with Cell Tracker Green and then incubated for 24 hours with or without autologous PBMC in addition to iRBC or uRBC in a 1:3 ratio. PBMC alone were cultured in parallel to ascertain the donor's NK response with iRBC. Analysis was done as described in Figure 6.8. This table summarizes the results for 3 donors tested with uRBC negative control values already subtracted.

* = no IFN- γ^+ NK cells detectable.

6.4 Discussion

The experiments in this chapter demonstrate that direct contact between the NK cell and the parasitized erythrocyte does occur and seems to be necessary for the NK cell to reach its maximum potential of IFN- γ production. The conjugation experiments showed that contact is transient, but that it consistently occurs in all donors tested. These data support the findings of Orago *et al* (1991) who demonstrated killing of iRBC by NK cells, a phenomenon which by definition requires cell:cell interaction (Orago *et al.*, 1991). The fact that NK cells formed conjugates with unparasitized erythrocytes also supports their finding of NK lysis of uRBC, albeit to a much lesser extent than that of iRBC, implying that a) non-MHC ligands for NK cells may exist on normal RBC, and b) that “neoantigens” expressed once the erythrocyte becomes parasitized augment the NK cell attraction. Saxena *et al* (1989) revealed that incubating PBMC with *P. falciparum* infected erythrocytes resulted in a subsequent diminished lysis of the K562 NK-sensitive line by the PBMC preparation, an effect not seen when uRBC were used instead (Saxena *et al.*, 1989). They hypothesized that the “neoantigens” on the surface of the iRBC serve to engage the NK cells thereby rendering them unable to interact with the K562 cells.

The uRBC-NK interaction may be of a very transient nature, possibly becoming stabilized by an additional interaction once the erythrocyte is parasitized. This second stabilizing interaction could be mediated through a *P. falciparum* “neoantigen” or alternatively, a host ligand that is abnormally exposed on the surface of the iRBC. These possible scenarios stem from the fact that the iRBC surface morphologically differs from the uRBC surface in that several parasite antigens as well as normally concealed host antigens become expressed (reviewed in Craig *et al.*, 2001 and Oh *et al.*, 1997). An example is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which plays a central role not only in antigenic variation but also in adhesion of infected erythrocytes to the endothelium, thereby facilitating parasite sequestration. Several host molecules have been identified as binding targets for PfEMP1, most of which are found on endothelial cells. However, CD31 (PECAM-1) has been implicated in PfEMP1 interactions (Chen *et al.*, 2000) and can also be found on NK cells (Poggi *et al.*, 1996), presenting evidence of a possible recognition pathway between parasites and NK cells.

Interestingly, the transwell experiments revealed that this contact is not only necessary for NK activation in terms of lysis (as assumed by Orago *et al*) but also in terms of IFN- γ production. The antiIL-12 and antiIL-18 experiments described in Chapter 5, showed that the quality of the cytokine milieu drastically affected NK IFN- γ release. The data collected here extends this observation to include the antigenic environment as well, in that NK cells not able to directly interact with the parasites showed impaired IFN- γ production.

Conversely, contact alone does not seem to be enough to induce maximum NK activation. Purified NK clones and cell lines of individuals previously categorized as high responders, were unable to make IFN- γ above background levels when cultured with iRBC. This lack of response was reversed with the addition of autologous PBMC showing that the purified NK lines and NK clones were still able to respond to iRBC. Although the clone-derived IFN- γ increased significantly under these conditions, the amount was not restored to the “normal” level seen with the non-purified NK cultures. This may be explained by a relative deficiency of IL-12/IL-18 producing cells in these cultures compared to normal PBMC cultures or may be due to the lack in the NK cell line (which contains only 3 clones) of the major IFN- γ -producing clones present in the PBMC culture (i.e. the line only contains a few of the different NK cell phenotypes present in whole blood).

The dual dependence of NK cells on cytokines (IL-12, IL-18 and possibly others) and direct parasite contact suggests that *P.falciparum* stimulation of this cell type involves a minimum of two different signals both of which need to be present to result in maximum activation. These data, combined with the observation of donor heterogeneity in NK IFN- γ release, suggest that NK cell recognition of its target in malaria infection may be partially mediated through a cellular receptor not found in all donors, or not expressed on all NK clones. The correlation of NK cell surface phenotype with IFN- γ production is the focus of experiments in the following chapter.

7.1 Introduction

In the previous chapter, I showed that there is contact between NK cells and parasitized erythrocytes, and that this contact is necessary for maximal IFN- γ production. Coupling this observation with the fact that I saw significant heterogeneity among donors in their ability to mount an NK-derived IFN- γ response, I arrived at the hypothesis that a ligand, present on infected but not uninfected erythrocytes, may interact with a specific NK receptor either not present in all individuals or differentially expressed in different people. The idea of direct pathogen recognition by NK receptors is supported by experiments with CMV where a viral MHC Class I homologue inhibits NK lysis of the CMV infected cell (as previously discussed in 6.1).

NK cell receptors can be divided into two groups, one belonging to the C-type lectin family and the other to the immunoglobulin (Ig) superfamily. C-type lectin receptors are non-polymorphic, and can be either inhibitory or activating. The inhibitory ones exist in the form of heterodimers pairing CD94 with NKG2A and B. A ligand for this group of receptors has been identified as HLA-E, which is a non-classical HLA Class I molecule (Braud *et al.*, 1998). The activating C-type lectin receptors include NKG2C, NKG2E as well as NKG2D, which - unlike the rest of the NKG2 gene products - forms NKG2D:NKG2D homodimers and associates with the DAP10 adaptor protein to induce signaling. MIC-A and MIC-B, stress inducible nonclassical HLA molecules expressed – for example – on many tumor cells have been identified as ligands for NKG2D, interaction with which leads to NK cell activation (reviewed in Moretta *et al.*, 2001). The activating heterodimers formed with NKG2C and NKG2E associate with DAP12, an accessory molecule which has immunoreceptor tyrosine-based activating motifs (ITAMs). DAP12, in turn, transmits an activating signal by becoming tyrosine phosphorylated following ligation of the receptor (reviewed in Moretta *et al.*, 2001). An additional group of non-polymorphic receptors that are not lectin-like but are

immunoglobulin-like, are the natural cytotoxicity receptors NKp30, NKp44 and NKp46. Natural ligands for NKp30, NKp44 and NKp46 have yet to be identified, although a recent paper suggests that NKp30 may interact with ligands on dendritic cells (Ferlazzo *et al.*, 2002).

The polymorphic immunoglobulin-like family of molecules are called killer Ig-like receptors (KIR) encoded by genes on human chromosome 19 as part of the leukocyte receptor complex. These receptors can be either activating or inhibitory depending on the morphology of their intracellular domain. Inhibitory KIR have long intracellular tails with immunoreceptor tyrosine-based inhibition motifs (ITIMs). ITIMs recruit SHP-1 and possibly SHP-2 phosphatases which cause the transmission of an inhibitory signal. Conversely, activating KIR have short cytoplasmic tails lacking ITIM motifs. As with the lectin-like activating receptors, activating KIR associate with the accessory molecule DAP12 (reviewed in Raulet *et al.*, 2001) (Figure 7.1). The nomenclature of KIR derives from both their intracellular and extracellular morphology, combining the number of Ig domains, the length of the cytoplasmic tail and the order in which its gene was identified. For example, 2DL3 describes a receptor with two Ig domains (2D) and a long inhibitory cytoplasmic tail (L) which was the 3rd such receptor to be identified.

HLA Class I molecules have been identified as ligands for inhibitory as well as activating KIR. However, activating KIR bind HLA Class I with a much lower affinity than their inhibitory counterparts (Vales-Gomez *et al.*, 1998) (Vales-Gomez *et al.*, 1999). Given this phenomenon, it has been hypothesized that these receptors may have evolved to recognize pathogen-specific ligands (Raulet *et al.*, 2001). This possibility is further supported by the plasticity of the KIR gene locus and the significant heterogeneity of the resulting KIR genotypes among individuals. Typing of the KIR locus has revealed extensive diversity in both KIR genotype and haplotype among individuals of different ethnicities (Uhrberg *et al.*, 1997) (Shilling *et al.*, 2002). Haplotypes may contain differing numbers of genes and there is extensive allelic diversity at each locus. Two general haplotypes have been defined: group A haplotypes contain 2DS4 as their only activating KIR whereas group B haplotypes have a wider variety of activating KIR. Both haplotypes include three 'framework' genes that are present in all individuals, namely 3DL3, 2DL4 and 3DL2 (Figure 7.2). However, exceptions to this rule have been

identified. In some haplotypes, 2DS4 alleles are mutated. Certain donors have been found to be homozygous for the mutated 2DS4 allele and, as such, express no functional activating KIR. If translated, this mutated gene product would result in a truncated soluble protein of unknown (if any) function (Hsu *et al.*, 2002).

It has been hypothesized that differences in KIR genotypes between individuals represent rapid evolution, possibly due to selective forces imposed by pathogens (McQueen *et al.*, 2002). It is, therefore, reasonable to conjecture that *Plasmodium* parasites - which have been proven to drive the genetic selection of haemoglobinopathies and numerous other polymorphisms, including variation in genes with known immune functions (Hill 1999) - may also be providing pressure for the diversification of NK receptors.

Mature human erythrocytes do not express MHC on their surface, therefore it is highly unlikely that a malaria-derived NK ligand would act as an MHC Class I decoy. However, KIR are the only known family of NK receptors known to be polymorphic, albeit only known to be triggered through MHC Class I for inhibitory KIR, with no identified ligand (as of yet) for the activating KIR. It is possible that iRBC express either a parasite-derived or an abnormally expressed self molecule that is a ligand for an activating NK receptor (either an activating KIR or an activating lectin-like receptor). More specifically, three hypotheses arise from this assumption: a) that high responders (as defined by their NK-derived IFN- γ response to iRBC) have more activating KIR (i.e. B haplotypes) than low responders (i.e. A haplotypes), b) that high responders have specific activating KIR, and c) that high responders have specific alleles of these activating KIR. Alternatively, this heterogeneity could also be a result of variable expression of the nonpolymorphic lectin-like or natural cytotoxicity receptors. These hypotheses are the focus of experiments described in this chapter.

7.2 Materials and Methods

7.2.1 KIR genotyping

Genomic DNA was prepared according to the protocol described in 2.13 and sent to the laboratory of Dr. Peter Parham at Stanford University, USA. The KIR genotyping for 27 donors was done by Dr. Karina McQueen and Ms. Nathalie Cheng using PCR-based sequence specific primers for the 16 KIR genes and pseudogenes. Table 7.1 summarizes the forward and reverse primers and the amounts used for the reaction. The PCR was done using hot-start procedures in an oil-free thermal cycler (PE9600 or PE9700 with PE9600 emulation mode, Applied Biosystems, Foster City, CA, USA). An initial denaturation of 100 ng DNA at 95°C for 2 minutes was followed by 10 cycles of 20 seconds at 94°C, 10 seconds at 65°C and 90 seconds at 72°C. This sequence was immediately followed by 20 cycles of 20 seconds at 94°C, 20 seconds at 61°C and 90 seconds at 72°C. A 7 minute final extension was done at 72°C and then the reaction mixture was cooled to 4°C for overnight storage to avoid product degradation. The existence of a KIR gene was determined by the presence or absence of a band of expected size on a 0.9% agarose gel (Life Technologies, Rockville, MD, USA) on which the PCR products were run at 100 V for 60 minutes and visualized by ethidium bromide staining. Growth hormone was a positive internal control for each PCR reaction.

7.2.2 KIR phenotyping

To determine KIR and C-type lectin receptor expression on IFN- γ ⁺ and IFN- γ ⁻ NK cells, 4 color flow cytometry was used combining the following fluorochromes: fluorescein (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridinin chlorophyll protein (PerCP). PBMC and parasite antigen preparations were made as described in sections 2.4-2.6. PBMC were cultured for 24 hours with 3×10^6 iRBC or uRBC before being assayed. The protocol for flow cytometry was followed as described in 2.8, with the exception that the staining antibodies used are shown in Table 7.2. Cells were analyzed using Becton Dickinson's FACS Calibur and CELLQuest software. A total of 100,000 events were collected from each sample for analysis.

The NKp30, NKp44, NKp46 (kind gift from A. and L. Moretta) and NKG2D (R&D) antibodies were unconjugated. The protocol for the surface staining was therefore slightly different. Cells were first stained for 30 minutes at 4°C with each unconjugated antibody (1µg/100 µl cell suspension) followed by a wash in FACS buffer. Subsequently, rabbit anti mouse PE labeled IgG1 (Sigma) was added (1µg/ 100 µl cell suspension) for another 30 minutes at 4°C in the dark followed by three washes in FACS buffer. At this point the surface staining for CD56 and CD3 and the intracellular IFN-γ was done as previously described.

specificity	forward primer			reverse primer			amplicon length (bp) ²
	name	primer conc pmol/μl	sequence (5'<---3'<)	name	sequence (5'<---3'<)	final conc pmol/μl	
KIR2DL1	Fe622	2.5	ccatcaagcagatgacg	Ra657	ccactgatggagagcat	0.1	1903
KIR2DL2	Fe638	5.0	acttctcttgcacagagaa	Ra654	gacctgcagagactaca	1.0	1868
KIR2DL3	Fg560	10.0	acagagaaagggaagttaag	RconB64	ccctgcagaggaactaag	1.0	1858
KIR3DL1	Fg1158	3.0	cdttcatagctggctgctg	Rt1375	caaggagacatdtggatca	0.3	812
KIR3DL2	R624	12.5	ccatttggctccatgatgt	Rc723	agagagaaaggtttctatctg	2.5	1690
KIR2DS1	R627	2.5	cggctccdtgatgactgt	Rc962	gaccacacagcagggcag	0.1	1948
KIR2DS2	R621	12.5	ttctccatcagctcagatgat	Ra899	aggggcacagaggaaagt	2.5	1839
KIR2DS2	Fe546	5.0	tyrtaacagagagggaagta	Rt767	ccagctdttctctgcaaa	1.0	1775
KIR2DS3	R682	12.5	tcactcccccatacagttt	Ra925	gcatctgtaggtctactct	2.5	~1800
KIR2DS4	Fe439	5.0	ctgggcctcccaagttca	Rg876	ggaaatgttcagttgatgc	1.0	1981
KIR2DS5	Fe561	5.0	agagaaggggacgthaac	R939	ggaaagagccgaaacatc	1.0	1952
KIR3DS1	Fg289	5.0	ggcagatatcttcagagag	Rt539	aggggtcttagagatcca	1.0	1885
KIR2DL4	Rgc157	5.0	aggacaaagccdtatgc	R939	ggaaagagccgaaacatc	1.0	1395
KIR2DL5	Rt196	5.0	tgacctgcagaggaagcat	Ra739	ccgggtgggctgaaggt	1.0	1151
KIR3DL3	R53	5.0	tggtcagggaacatccctt	Ra669	ccgacacatctatagggtta	1.0	1824
KIR3DP1	Fcon80	7.6	ccatgtgcgtcatgtgtcg	Rc269	ctgacacaccagtgaggga	1.0	361/1834
	Fcon113	7.6	agggtctttttgtctgc				1047
KIR2DP2	FA674		ttctttactactaccacca	R939	ggaaagagccgaaacatc	1.0	1825

Table 7.1: KIR genotyping. This table summarizes the 16 KIR genes and pseudogenes and the primers used to genotype by PCR.

Antibody	target	manufacturer	fluorochrome	isotype
KARp50.3	2DS4	Beckman Coulter	PE	IgG2a
Z27	3DL1,3DL2	Beckman Coulter	PE	IgG1
EB6 (CD158a)	2DL1,2DS1	Beckman Coulter	PE	IgG1
DX27 (CD158b)	2DL2,2DL3,2DS2	Becton Dickinson (Oxford, UK)	PE	IgG2a
NKB1	3DL1	Becton Dickinson	PE	IgG1
CD94	CD94 dimers (NKG2A,B,C)	Beckman Coulter	PE	IgG2a
NKG2A	NKG2A	Beckman Coulter	PE	IgG2b
CD3	CD3	Becton Dickinson	PerCP	IgG1
CD56	CD56	Becton Dickinson	APC	IgG1
IFN- γ	IFN- γ	Pharmingen (Cowley, UK)	FITC	IgG1
NKG2D	NKG2D homodimers	R&D	unlabeled	IgG1
NKp30	NKp30	Moretta	unlabeled	IgG1
NKp44	NKp44	Moretta	unlabeled	IgG1
NKp46	NKp46	Moretta	unlabeled	IgG1
isotype control	-	Caltag	PE	IgG1
isotype control	-	Caltag	PE	IgG2a
isotype control	-	Caltag	PE	IgG2b
isotype control	-	Becton Dickinson	PerCP	IgG1
isotype control	-	Becton Dickinson	APC	IgG1
isotype control	-	Becton Dickinson	FITC	IgG1

Table 7.2: Fluorescence-labeled antibodies. This table summarizes the antibodies used for flow cytometric analysis in the experiments described in this chapter as well as the relevant isotype controls.

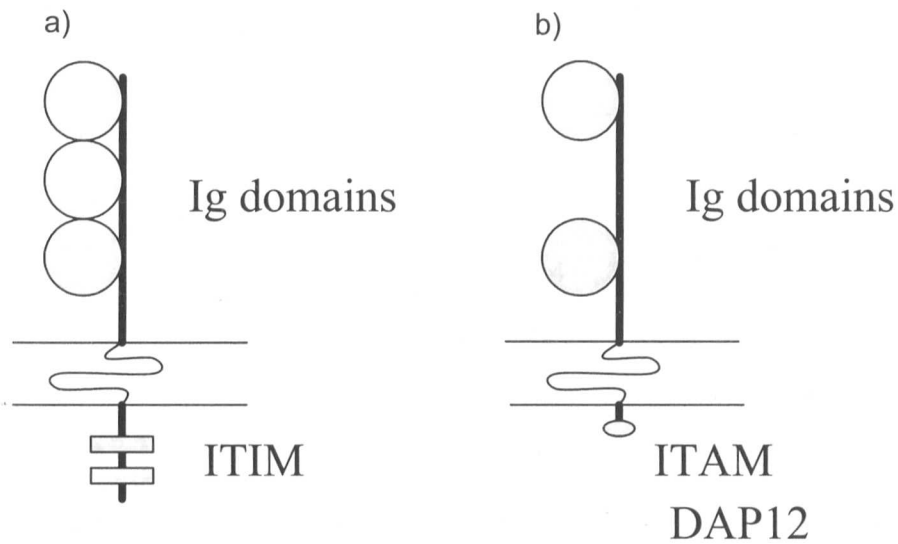


Figure 7.1: Inhibitory and activating Killer Immunoglobulin-Like Receptors (KIR). KIR can have 2 or 3 extracellular immunoglobulin domains and can be inhibitory or activating. Inhibitory KIR (a) have a long intracellular tail with signal-blocking ITIM motifs, whereas activating KIR have short tails with a charged residue in their transmembrane domain which allows them to associate with the ITAM-containing adaptor signaling molecule, DAP12, to successfully transmit a signal into the cell.

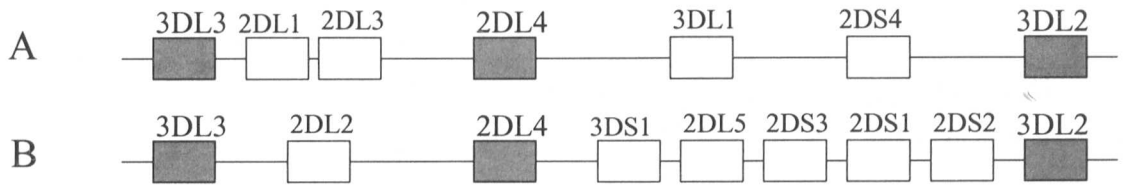


Figure 7.2: KIR haplotypes. Individuals can be categorized as having A or B KIR group genotypes. An example of each is shown. The genes shown in gray represent framework genes present in all individuals. A genotypes also include genes for 2DL1, 2DL3, 2DS4 and 3DL1 whereas B genotypes are characterized by 2DL5, and typically contain more activating KIR. An individual can have an AA, a BB or an AB haplotype.

7.3 Results

7.3.1 Correlating NK-derived IFN- γ with KIR genotype

DNA samples from each donor were sent to Karina McQueen at Stanford who proceeded to genotype each donor's KIR. At this point in time, KIR analysis tells us only whether the gene is present or not and whether it encodes a full length (i.e. functional) KIR or a truncated (non-functional) receptor. Further analysis (yet to be completed) will reveal the extent of allelic polymorphism within these genes. The first hypothesis of interest was to correlate the level of an individual's NK-derived IFN- γ response to iRBC with the presence of more than one activating KIR, in other words with a B haplotype. In order to best visualize an association, donors were grouped in Table 7.3 according to the percentage of NK cells staining positive for IFN- γ . High responders had over 10% of NK cells producing IFN- γ , low responders had under 1%, and intermediate responders had anything in between, with the majority falling between 2-3%. The last column in the table states the donors' KIR haplotype. The presence or absence of each KIR gene is depicted by a shaded or a white box, respectively.

Of the 27 donors, 6 had the AA genotype (i.e. have only the 2DS4 activating KIR, although they may express two different alleles of 2DS4), 19 were AB heterozygotes and one donor was a BB homozygote. We looked to see whether there was any difference in the frequency of A and B haplotypes between European and African donors, which might indicate selection for the presence or absence of activating KIR (the three Asian donors were excluded from the analysis). Among the 14 European donors there were 17 A haplotypes and 11 B haplotypes (frequency of A=0.61; frequency of B=0.39) and among the 10 African donors there were 11 A haplotypes and 9 B haplotypes (frequency of A=0.55; frequency of B=0.45). This suggests that the B haplotype (with more activating KIR) may be more prevalent among African donors, possibly reflecting higher pathogen challenge, but the difference was not statistically significant on this small number of donors ($\chi^2=0.16$, $df=1$, $p>0.5$). As expected, the three framework genes 3DL3, 2DL4 and 3DL2 were detected in all donors. 2DL3 was also detected in all donors in this group.

Table 7.3: KIR genotypes. This table summarizes the KIR genotype of 27 donors. A shaded box signifies the presence of a gene whereas a white box signifies its absence. Donors are organized based on level of NK response to iRBC, with the first column stating the percentage of IFN- γ + NK cells after 24 hour iRBC stimulation with uRBC negative control already subtracted. This value is derived from a representative experiment for donors that were assayed multiple times. The last column in the table states each donor's genotype as determined from their KIR haplotype.

% IFN- γ NK cells	DONOR	ETHNICITY	KIR GENES																Haplotype
			2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1	3DP1	
22.1	FMO	African																	AB
20.8	094M	Asian																	AB
24.2	063M	European																	AB
14	JET	European																	AB
13.4	093F	European																	AA
11.8	073F	European																	AA
10	HTD9	African																	AB
3.5	TH	European																	AB
2.8	AMSP	part-African																	BB
2.2	HTD1	African																	AA
2.8	HTD5	African																	AB
2.8	096F	European																	AB
1.7	HTD7	European																	AB
3.8	AJH	European																	AB
2.4	CS	European																	AB
2	KAT	European																	AB
2	MH	European																	AB
1.2	RaT	Asian																	AA
2.4	SS	European																	AB
0.1	014F	European																	AB
0.1	HTD8	African																	AB
0.2	HTD6	African																	AA
0.4	JC016F	European																	AA
0.9	HT	African																	AB
0.3	BdS	Asian																	AB
0.6	KT	African																	AB
0.2	MJ	African																	AB

The inhibitory KIR 2DL1 and 3DL1 were found in all but one donor (with the BB genotype) and 2DL2 and 2DL5 were detected in 17 and 14 donors, respectively. As expected, 2DL5 was indicative of a B haplotype. The number for activating KIR ranged from 1 to 6 (in 094M).

It immediately becomes apparent that high responders do not fall exclusively into the B haplotype category, with the majority being AA and the rest AB. The mean number of activating KIR per person did not differ between groups (mean=2.57, 2.50 and 2.25 in high, intermediate and low responder groups, respectively) and the prevalence of the B haplotype was 0.714 in high responders, 0.833 in intermediate responders and 0.750 in low responders, suggesting a trend for B haplotypes to be correlated with low NK responses. This point is illustrated further in Figure 7.3 where the IFN- γ + NK cell percentages are plotted against numbers of activating KIR (as shown in Table 7.3) for each donor. With the exception of a single outlier, the donors seem to roughly fall into two groups as indicated by the dotted lines. It seems that donors who have low numbers of activating KIR are more likely to make a strong NK-derived IFN- γ response to iRBC. However, this difference in IFN- γ response cannot be explained through the consistent presence of a specific activating KIR, as was the premise of the hypothesis discussed in section 7.1, but it is still possible that specific alleles of activating KIR may be associated with IFN- γ production. This hypothesis can only be properly tested in a much larger study.

7.3.2 Correlating NK IFN- γ production with KIR phenotype

An alternative approach for looking at associations between NK receptor expression and responses to iRBC, is to look by flow cytometry at NK receptor expression on responding NK cells. Therefore, PBMC were stimulated with iRBC or uRBC for 24 hours and then NK cells were analyzed by 4 color FACS for expression of IFN- γ and a panel of NK receptors (outlined in Table 7.2). Since the antibodies currently available for KIR phenotyping recognize somewhat conserved epitopes on the receptors, it is only possible to identify groups of receptors as opposed to individual ones. Examples

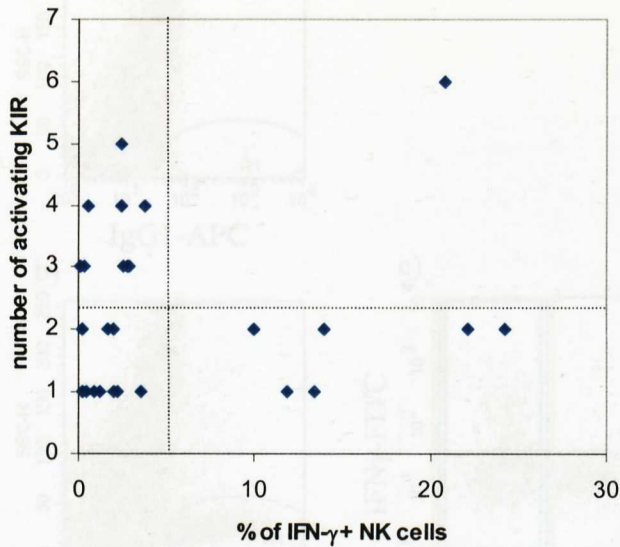


Figure 7.3: Correlation on NK IFN- γ production and number of activating KIR.

The percentage of IFN- γ + NK cells after 24 hour iRBC stimulation (with uRBC negative control already subtracted), derived from a representative experiment for donors that were assayed multiple times, is plotted against the number of activating KIR genes present in each donor. Each point on the graph corresponds to a different donor.

Figure 7.4: NK receptor phenotyping – example of analysis. All CD56+ cells were gated (b) and examined for CD3 and IFN- γ expression (c). Receptor analysis was done drawing from all IFN- γ + NK cells (blue gate), all IFN- γ - NK cells (green gate) or all NK cells (orange gate). The subsequent PE analysis is illustrated using CD94 as an example drawing from each of the three gates (d, e, f). The isotype control is also shown (a).

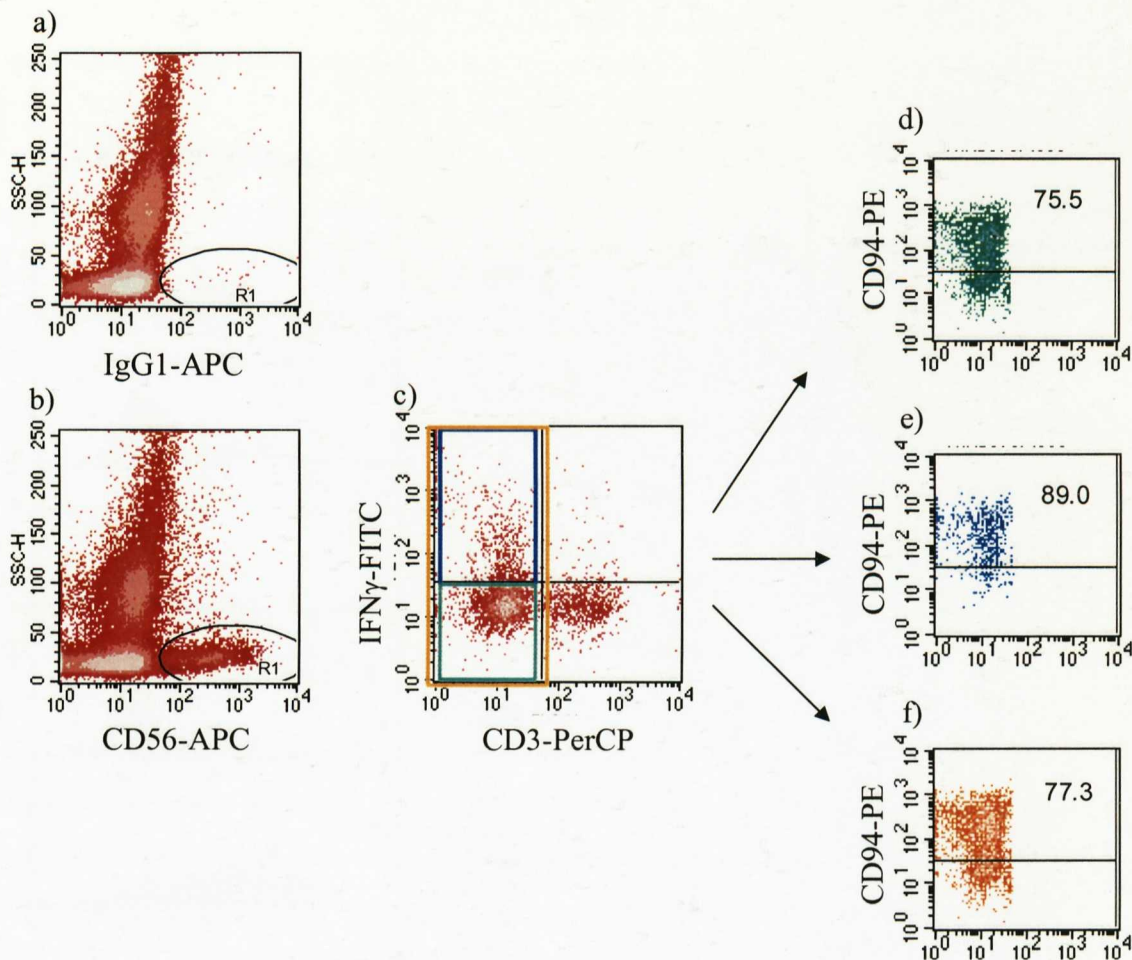
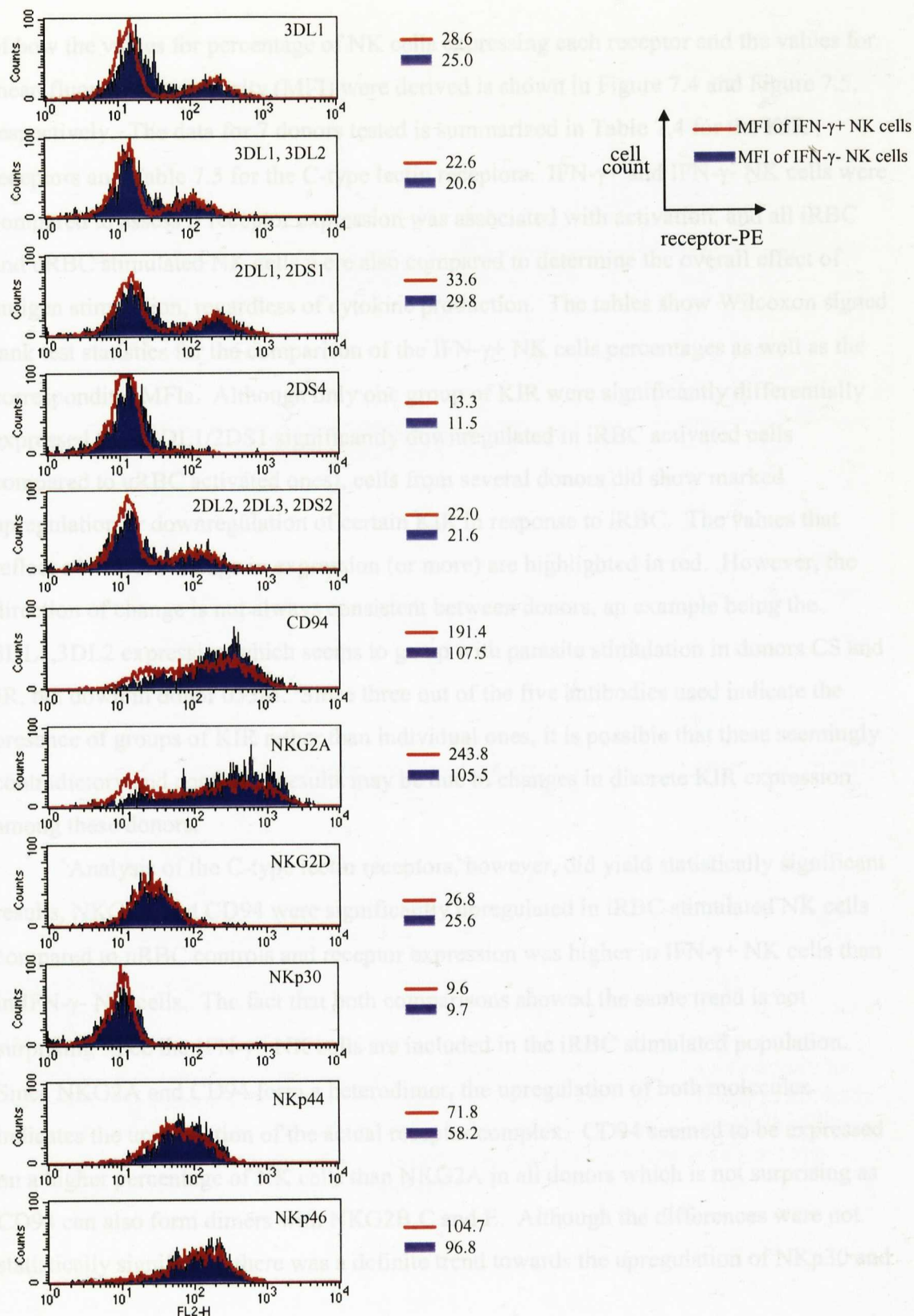


Figure 7.4: NK receptor phenotyping – example of analysis. All CD56+ cells were gated (b) and examined for CD3 and IFN- γ expression (c). Receptor analysis was done drawing from all IFN- γ + NK cells (blue gate), all IFN- γ - NK cells (green gate) or all NK cells (orange gate). The subsequent PE analysis is illustrated using CD94 as an example drawing from each of the three gates (d, e, f). The isotype control is also shown (a).

Figure 7.5: Example of Mean Fluorescence Intensity (MFI) Analysis in one donor. Each plot in this figure illustrates the staining for each individual NK receptor tested. The PE channel (receptor stain) is plotted against event count, and the cells drawn from the IFN- γ + NK gate are in purple and are overlaid onto the cells drawn from the IFN- γ - NK gate which are depicted in red. Since the event counts were different for the two gated populations, they were scaled within the Cellquest program to make them equal in order to better depict the MFI shifts. MFI values are shown for each histogram next to the corresponding plot.



of how the values for percentage of NK cells expressing each receptor and the values for mean fluorescence intensity (MFI) were derived is shown in Figure 7.4 and Figure 7.5, respectively. The data for 7 donors tested is summarized in Table 7.4 for the KIR receptors and Table 7.5 for the C-type lectin receptors. IFN- γ ⁺ and IFN- γ ⁻ NK cells were compared to assess if receptor expression was associated with activation, and all iRBC and uRBC stimulated NK cells were also compared to determine the overall effect of antigen stimulation, regardless of cytokine production. The tables show Wilcoxon signed rank test statistics for the comparison of the IFN- γ ⁺ NK cells percentages as well as the corresponding MFIs. Although only one group of KIR were significantly differentially expressed (KIR2DL1/2DS1 significantly downregulated in iRBC activated cells compared to uRBC activated ones), cells from several donors did show marked upregulation or downregulation of certain KIR in response to iRBC. The values that reflect a two-fold change in expression (or more) are highlighted in red. However, the direction of change is not always consistent between donors, an example being the 3DL1,3DL2 expression which seems to go up with parasite stimulation in donors CS and JR, but down in donor 053M. Since three out of the five antibodies used indicate the presence of groups of KIR rather than individual ones, it is possible that these seemingly contradictory and confusing results may be due to changes in discrete KIR expression among these donors.

Analysis of the C-type lectin receptors, however, did yield statistically significant results, NKG2A and CD94 were significantly upregulated in iRBC stimulated NK cells compared to uRBC controls and receptor expression was higher in IFN- γ ⁺ NK cells than in IFN- γ ⁻ NK cells. The fact that both comparisons showed the same trend is not surprising since the IFN- γ ⁺ NK cells are included in the iRBC stimulated population. Since NKG2A and CD94 form a heterodimer, the upregulation of both molecules indicates the upregulation of the actual receptor complex. CD94 seemed to be expressed on a higher percentage of NK cells than NKG2A in all donors which is not surprising as CD94 can also form dimers with NKG2B,C and E. Although the differences were not statistically significant, there was a definite trend towards the upregulation of NKp30 and

		iRBC		iRBC					
		IFN- γ + NK	IFN- γ - NK	IFN- γ + MFI	IFN- γ - MFI	iRBC	uRBC	iRBC MFI	uRBC MFI
3DL1	CS					10.4	5.3	16.7	11.2
	TH					36.8	35.5	48.2	50.6
	FMO	18.0	19.6	121.7	19.4	19.1	20.0	14.8	13.1
	JR	8.8	6.4	16.6	13.7	5.1	3.9	13.8	12.3
	073F	38.4	35.9	47.7	48.1	35.8	38.5	47.2	49.5
	093F	31.5	27.8	28.6	25.0	28.3	25.9	25.7	18.5
	053M	38.9	19.5	40.7	23.2	23.0	20.1	25.4	18.3
	z=	1.75		1.75		1.35		1.18	
	p=	0.08		0.08		0.18		0.24	
3DL1,3DL2	CS					2.2	0.9	12.0	9.9
	TH					36.1	36.4	39.5	42.3
	FMO	19.9	21.1	9.3	18.1	20.6	21.5	13.1	14.9
	JR	6.3	2.5	14.4	13.3	1.7	0.7	13.3	11.9
	073F	41.8	36.9	39.8	33.8	37.3	38.4	34.3	30.2
	093F	29.5	27.7	22.6	20.6	27.6	26.7	20.9	16.2
	053M	36.0	7.9	32.4	10.7	9.8	19.6	11.8	15.6
	z=	1.75		0.90		-0.17		0.50	
	p=	0.08		0.35		0.87		0.61	
2DL1,2DS1	CS					18.0	17.7	20.4	15.5
	TH					21.7	22.4	20.9	21.3
	FMO	25.1	36.3	10.5	26.6	36.5	42.9	20.7	13.1
	JR	42.3	49.9	59.9	56.8	52.0	57.1	56.8	58.0
	073F	41.0	24.8	34.4	20.1	29.4	34.3	21.7	19.3
	093F	38.3	34.1	33.6	29.8	38.7	39.5	30.6	23.0
	053M	35.7	12.2	32.2	15.8	19.6	26.1	15.8	17.2
	z=	0.67		0.94		-2.20		1.36	
	p=	0.50		0.35		0.03		0.18	
2DS4	CS					2.3	1.2	11.9	10.4
	TH					51.0	51.7	57.7	60.2
	FMO	35.6	50.8	17.7	62.4	46.0	53.0	43.0	25.9
	JR	8.5	1.5	16.5	11.7	1.4	0.6	11.7	10.1
	073F	5.4	3.8	11.0	10.3	4.0	2.7	10.5	8.1
	093F	7.0	2.6	13.3	11.5	3.3	3.7	11.8	8.1
	053M	65.0	52.5	72.0	55.2	54.1	54.0	61.1	57.2
	z=	0.67		0.67		0.34		1.70	
	p=	0.50		0.50		0.74		0.09	
2DL2,2DL3,2DS2	CS					20.0	20.0	17.6	16.4
	TH					37.8	38.4	25.0	26.1
	FMO	9.8	22.3	11.4	13.9	21.0	26.8	11.4	8.9
	JR	50.8	51.7	31.9	31.4	53.7	57.2	31.4	32.3
	073F	27.9	27.4	18.9	18.7	29.2	34.1	18.7	18.6
	093F	30.3	30.4	22.0	21.6	32.5	36.9	21.7	19.8
	053M	52.5	42.8	50.5	37.0	46.4	43.5	38.7	34.7
	z=	-0.41		0.94		-1.80		1.50	
	p=	0.69		0.35		0.07		0.13	

Table 7.4: KIR expression. This table summarizes the NK KIR phenotyping data collected for 7 donors. The receptor (s) stained are listed on the far left for each panel of data. The first two columns compare the percent of IFN- γ + and IFN- γ - NK cells expressing each receptor or receptor group. The second pair of columns compare the corresponding mean fluorescence intensities (MFI). The third pair of columns compare the percent of iRBC and uRBC stimulated NK cells expressing each receptor and the fourth pair of columns compare the corresponding MFI. Wilcoxon signed rank test statistics are shown for each pair of columns.

		iRBC		iRBC		iRBC	uRBC	iRBC MFI	uRBC MFI
		IFN- γ + NK	IFN- γ - NK	IFN- γ + MFI	IFN- γ - MFI				
NKG2A	CS					62.5	60.8	105.8	89.8
	TH					37.3	36.4	38.5	36.5
	FMO	82.9	53.9	119.6	76.9	62.5	47.1	119.6	72.8
	JR	56.5	48.5	74.5	57.1	47.5	41.2	56.9	43.5
	073F	77.5	68.0	177.0	112.9	68.1	49.7	120.5	53.7
	093F	88.4	68.8	243.8	105.5	72.1	45.9	125.4	47.8
	053M	87.7	72.4	320.5	157.6	74.5	61.1	117.5	98.9
	z=	2.02		2.02		2.37		2.37	
	p=	0.04		0.04		0.02		0.02	
CD94	CS					64.8	68.0	71.1	74.0
	TH					55.9	50.5	53.9	46.8
	FMO	88.3	56.8	159.6	49.6	63.6	49.5	62.8	45.6
	JR	76.2	60.9	94.0	53.8	58.4	49.2	54.0	44.4
	073F	89.0	76.7	152.6	95.7	77.3	54.3	102.0	49.6
	093F	92.3	79.7	191.4	107.5	81.1	64.0	120.9	63.3
	053M	90.3	89.1	216.0	139.9	88.5	82.7	148.6	107.5
	z=	2.02		2.02		2.20		2.20	
	p=	0.04		0.04		0.03		0.03	
NKp30	CS								
	TH								
	FMO								
	JR	4.0	0.3	20.4	10.6	0.7	1.4	10.6	8.9
	073F	1.3	0.6	9.0	8.7	1.1	1.2	8.7	7.4
	093F	1.3	0.4	9.6	9.7	1.4	3.4	9.7	7.7
	053M	65.2	11.5	21.5	10.6	30.5	39.3	13.4	14.7
	z=	1.80		1.46		-1.80		1.10	
	p=	0.07		0.14		0.07		0.27	
NKp44	CS								
	TH								
	FMO								
	JR	90.5	87.0	95.1	69.7	92.3	86.6	69.8	42.6
	073F	89.1	83.9	85.2	65.6	89.7	91.3	68.0	60.4
	093F	87.2	80.7	71.8	58.2	88.3	86.1	61.2	50.6
	053M	94.4	75.6	49.5	28.2	82.1	83.1	33.7	36.0
	z=	1.83		1.83		0.73		1.50	
	p=	0.07		0.07		0.47		0.14	
NKp46	CS								
	TH								
	FMO								
	JR	80.0	95.4	80.0	105.6	97.3	98.8	105.5	110.1
	073F	89.7	87.6	123.5	117.5	90.2	95.1	144.0	116.5
	093F	91.4	89.0	104.7	96.8	91.7	91.7	97.5	85.9
	053M	94.7	59.3	58.3	25.0	71.2	91.2	33.1	77.4
	z=	0.73		0.73		-1.83		0.00	
	p=	0.47		0.47		0.07		1.00	
NKG2D	CS								
	TH								
	FMO								
	JR	73.7	80.8	36.4	55.1	89.0	95.9	55.2	49.1
	073F	62.9	55.6	43.1	33.8	68.5	76.4	30.8	34.7
	093F	39.1	40.0	26.8	25.6	55.0	79.1	25.9	30.3
	053M	94.3	58.0	32.7	17.8	69.1	73.6	21.4	25.1
	z=	0.73		0.37		-1.83		-0.37	
	p=	0.47		0.72		0.07		0.72	

Table 7.5: C-type lectin receptor expression. This table summarizes the NK C-type lectin receptor phenotyping data collected for 7 donors. The receptors stained for are listed on the far left for each panel of data. The first pair of columns compare the percent of IFN- γ + and IFN- γ - NK cells expressing each receptor or receptor group. The second pair of columns compare the corresponding mean fluorescence intensities (MFI). The third pair of columns compare the percent of iRBC and uRBC stimulated NK cells expressing each receptor and the fourth two columns compare the corresponding MFI. Wilcoxon signed rank test statistics are shown for each pair of columns.

NKp44 in IFN- γ ⁺ NK cells (highlighted in red) as determined by comparisons of IFN- γ ⁺ NK cell percentages and MFI, respectively.

7.4 Discussion

The experiments in this chapter attempted to correlate a donor's level of NK-derived IFN- γ in response to iRBC with their NK surface phenotype. Although the number of donors tested was small – and possibly too small to reveal significant associations – the results do show some interesting trends that can be followed up in larger studies. However, I was able to reject the hypothesis that low responders and high responders are divided into A and B haplotypes, respectively. This does not mean that there is no link between KIR genotype or phenotype and malaria responsiveness, since it is still quite possible that the frequency of a particular activating KIR or a specific KIR allelic variant may be significantly higher in responding individuals. Much larger genetic population studies need to be conducted in order to adequately address these questions.

The four color flow cytometry also did not reveal any association between KIR expression and malaria responses on the seven donors that were tested. However, this analysis is of limited value as the only antibodies that are available for such studies do not differentiate between individual KIR genes and do not detect allelic polymorphisms. Rather, the antibodies indicate the presence of KIRs sharing conserved epitopes. With the development of more specific reagents or methods of typing (e.g. RT-PCR) associations with responses to pathogens may emerge.

Interestingly, two lectin-type receptors were upregulated in activated (i.e. IFN- γ +) NK cells, namely CD94 and NKG2A. As NKG2 isoforms dimerize with CD94, the parallel upregulation of NKG2A and CD94 suggests that the CD94:NKG2A heterodimer is upregulated on activated cells. CD94:NKG2A has been identified as an inhibitory receptor, rescuing target cells from lysis by binding of non-classical MHC Class I molecules (HLA-E in humans and Qa-1^b in mice) (Raulet *et al.*, 2001). In murine NK cells, ligation of Qa-1^b by CD94:NKG2A inhibits cytotoxicity (Vance *et al.*, 1998) but MHC Class I-mediated inhibition of IFN- γ production is independent of CD94:NKG2A (in that IFN- γ production is downregulated in the presence of MHC Class I both in NK cells that are CD94:NKG2A-positive and in cells that fail to express this receptor (Kubota *et al.*, 1999). Further evidence that NK cytotoxicity and IFN- γ production are regulated by

different mechanisms comes from studies by Kurago *et al* who showed that human NK clones kill K562 and 721.211 target cells equally well, but that K562 cells induce much higher levels of IFN- γ production (Kurago *et al.*, 1998). A similar lack of correlation between cytotoxicity and IFN- γ production has been demonstrated in murine *Trypanosoma cruzi* infection where IFN- γ peaked at 24 hours but cytotoxicity persisted for 8 days (Une *et al.*, 2000).

However, these studies do not help us to answer the question of why an inhibitory receptor such as CD94:NKG2A might be upregulated on activated cells. One explanation might be that CD94:NKG2A serves a regulatory function in activated NK cells, its upregulation serving to limit the level of NK activation and thus prevent potential pathological effects of an overactive cytokine response. Further studies are required in order to assess the significance and function of these receptors in NK cell-malaria interactions.

Chapter 8 Final Discussion

8.1 Significance of study

Repeated attempts have been made to create a vaccine to protect against *P. falciparum* infection. However, an empirical approach to vaccine development - which has been successful for numerous other pathogens - has, so far, been unsuccessful against malaria. This failure has been mainly attributed to the parasite's highly developed ability to vary or mutate its antigenic components and to thereby evade the immune response. Much research has, therefore, refocused on attempting to elucidate exactly which parasite antigens are involved in triggering an immune response.

The nature of the ensuing immune response has also become central in understanding the disease, in that parasites can elicit different responses depending on the host's state of immunity. The fact that a state of clinical immunity does exist for malaria indicates that a) immunity is attainable and b) that if the mechanisms of immunity are understood, a vaccine designed to stimulate the immune system in similar ways could potentially confer protection to naïve individuals.

IFN- γ is one of the many factors involved in the immune response to *P. falciparum*. Its indispensable involvement in both pathology and protection during the course of infection makes its regulation a central point of interest. If quantity, location, source and timing of IFN- γ release can be correlated with protection or with pathology, a vaccine incorporating this component in its design could potentially be more efficacious in conferring immunity to the recipient.

As a contribution to solving this problem, the work presented in this thesis attempts to clarify and define the sources of, and kinetics of, IFN- γ production, and in the process identified NK cells as a potentially important player in the immune response to *P. falciparum*. Therefore, the focus of the work shifted towards defining the contribution of NK cells to IFN- γ production, including their mechanisms of activation and parasite recognition.

8.2 Key Findings

The first significant finding was that early (<48 hours) IFN- γ production is preferentially induced by live parasitized erythrocytes rather than dead schizont lysate. This suggests that in freeze-thawing of the parasite, a major antigenic component is eliminated which is responsible for directly and/or indirectly activating innate immune cells to release pro-inflammatory mediators. The greater antigenicity of live rather than dead parasites has also been demonstrated for *Leishmania* where live promastigotes induced significantly higher levels of IFN- γ than dead ones (Nylen *et al.*, 2001). The authors concluded that the stimulatory factors involved were not secreted but were part of the parasite membrane and could be accumulated and modified by a live parasite. This idea may be applicable to the *P. falciparum* system as well, since freeze-thawing schizonts results in fragmentation of the parasite, including its membrane. Sensitivity of cellular responses to antigen preparation has been further demonstrated in differential $\gamma\delta$ T cell proliferation to *Mycobacterium tuberculosis* (Batoni *et al.*, 1998). Although all *M. tuberculosis* antigens used were dead, the study revealed that the preparation method significantly affected the composition of the responding cell population, with the $\gamma\delta$ T cell subset reacting to bacteria killed by heating at 85°C. Furthermore, a recent study examining CD8 T cell responses, revealed greater proliferation of perforin-positive CD8 cells to live rather than dead Bacillus Calmette-Guerin (BCG) following *in vitro* culture (Tsunetsugu-Yokota *et al.*, 2002). Likewise, preferential activation of $\gamma\delta$ T cells has also been previously shown with *P. falciparum*, where only live parasites induced a proliferative response leading the authors to conclude that the pivotal parasitic component is a labile phospholipids, destroyed once the parasites are killed (Waterfall *et al.*, 1998).

The observation of preferential IFN- γ induction by live intact parasitized erythrocytes was subsequently extended to NK cells, with data showing that PfSL was unequivocally ineffective at eliciting an IFN- γ response from NK cells across all donors, whereas iRBC resulted in substantial NK-derived IFN- γ but only in certain donors.

The possibility that NK cells were indirectly activated (i.e. in a bystander manner) to produce IFN- γ was investigated next. The data indicated a strong IL-12 dependency and a lesser, but nonetheless evident, reliance on IL-18 for optimal IFN- γ responses.

Numerous studies have demonstrated NK cell dependence on the presence of IL-12 and IL-18 and, therefore, these results were not surprising (Micallef *et al.*, 1996) (Tomura *et al.*, 1998) (Fehniger *et al.*, 1999). However, the marked heterogeneity seen in donor NK reactivity could not be explained by inadequate amounts of IL-12 or IL-18, as exemplified by the failure of addition of these monokines to nonresponder cells to augment the IFN- γ response. Differences between responses to live and dead stimuli also could not be explained by differences in IL-12 or IL-18 production, suggesting that a component of the live parasite itself is involved in NK cell activation. However, there are other cytokines that are involved in NK cell activation (e.g. IL-2 and IL-15) that we have not investigated (Fehniger *et al.*, 1999) (Kogure *et al.*, 2002). It would, therefore, be of interest to determine whether or not individual differences in IL-2, IL-15 and other cytokines affect NK responses.

The hypothesis that a live parasite component is involved in NK cell activation was further supported by the transwell and conjugate experiments described in Chapter 6 revealing that contact does occur between the NK cell and the parasite and that this contact is necessary for optimal NK activation. These results, combined with data showing that cell lines were unable to produce IFN- γ in the absence of accessory cells, led to the hypothesis that NK cells need two signals to become activated: a (bystander) cytokine signal and a (direct) contact signal. Since the donor heterogeneity in NK responses could not be explained from differences in the cytokine signal, it was hypothesized that variation in the contact signal was responsible for the heterogeneity in the NK response. Considering that KIR are the only known polymorphic family of NK receptors, I arrived at the tentative hypothesis that KIR genotype and phenotype were behind these variations, with the wider goal of investigating the expression and involvement of as many NK receptors as possible.

KIR genotyping did not reveal any consistent patterns correlating to the donors' NK IFN- γ responses, but the donor pool was very small. However, the data did not support the hypothesis that high NK-IFN- γ producers have a greater number of activating KIR. In fact, the opposite tended to be the case; with one exception, donors who had the potential to express many activating KIR actually tended to have low NK responses. Likewise, KIR phenotyping also did not reveal any distinct patterns, possibly partly due

to the inability of the available antibodies to differentiate between different KIRs and the lack of allele specific reagents. However, the lectin-type receptors, CD94 and NKG2A, which form dimers, were significantly upregulated in the iRBC stimulated, IFN- γ producing cells. The upregulation of an apparently inhibitory receptor raises the possibility that NK activation is accompanied by initiation of mechanisms to prevent excessive activation of the cells and to allow a negative feedback mediated downregulation of the response. Similar systems of control have previously been described in various leukocyte populations, an example being upregulation of CTLA-4 on activated T cells. CTLA-4 is upregulated soon after T cell activation and seems to antagonize CD28, and possibly other activating T cell receptors, for B7 (or other) ligands thereby inhibiting cell proliferation (reviewed in Greenwald *et al.*, 2002). It is, therefore, possible that the CD94:NKG2A heterodimer serves a regulatory role in NK malaria-induced activation. Again, this would be an interesting avenue for future studies.

8.3 Implications of findings and future work

The central implication of the work described is that NK cells constitute a significant source of a vital inflammatory mediator in the response to human malaria and may represent an important component of the protective immune response against *P. falciparum*. It remains to be seen if individuals who have highly reactive NK cells are predisposed to more severe pathology or if they are more effectively protected against symptoms during a primary infection. Such a study could potentially be done in regions where transmission frequencies vary over small areas due to altitude, such as the highlands of East Africa and New Guinea, or in migrant settlements where naïve or relatively unexposed individuals settle in endemic areas, such as West Papua, Indonesia (Baird *et al.*, 1998). Such studies could initially determine NK responses through *in vitro* culture as described here, and then could prospectively follow individuals in order to look for associations between NK responses and infection or pathology following initial infection, or even over the course of several infections to see if NK responsiveness affects the speed with which clinical immunity is ultimately attained. The benefits of conducting these studies in a) malaria endemic regions and b) in areas of variable

transmission are manifold. Firstly, obvious ethical hurdles of experimental infection in humans are bypassed, and secondly, potentially confounding factors such as genetic variation and environmental exposure to other pathogens which could affect the development of the immune system, are also avoided.

Another benefit of prospective studies would be to gain further insight into the role played by KIR in NK responses to malaria. As mentioned in Chapter 7, much work still needs to be done on the genotyping of these receptors. In order to assess the selective pressure malaria may have exerted on the KIR locus, large scale prospective studies correlating genotype with NK cell *in vitro* activity as well as severity of incident infection also need to be conducted recruiting donors from various malaria endemic geographic locations. If KIR gene frequencies are correlated to levels of immunity, severity of pathology, or even just with level of exposure to malaria, specific alleles may be used as markers to predict an individual's response to infection and may also determine the course and nature of anti-malarial prophylaxis or remedies administered.

Within the realm of the experiments described here, the lectin-type receptors seem to pose a more immediate set of questions. The upregulation of CD94-NKG2A on activated NK cells begs the question of whether or not these receptors serve a function. It would be interesting to block them, for example with antibodies, and assess how this affects the NK response to *P. falciparum* in terms of IFN- γ production and cytotoxicity. Also, since anti-parasite cytotoxic activity has been previously demonstrated, it remains to be seen if this correlates with IFN- γ production and if these two effector functions are induced in a similar manner. There is evidence that CD56^{bright} and CD56^{dim} NK cell subsets have different levels of receptor expression, with CD56^{bright} having higher surface levels of CD94/NKG2 and lower levels of KIR than the CD56^{dim} population (Frey *et al.*, 1998). Furthermore, the CD56^{bright} subset has also been linked to higher cytokine production, lower cytotoxicity, lower proclivity to forming conjugates with target cells (Jacobs *et al.*, 2001) and seems to be costimulated to proliferate and to produce IFN- γ through ligation of CD94 (Voss *et al.*, 1998). Although NK cell subsets were not analyzed separately in the experiments described, it would be interesting to reexamine the data to see if iRBC-NK conjugate formation and iRBC-induced IFN- γ production can be correlated to the level of CD56 expression on NK cells. It would also be interesting to

examine the level of CD94 and NKG2A expression in IFN- γ + CD56^{bright} and IFN- γ + CD56^{dim} cells, as these results would better characterize the features of NK cell subsets responding in *P. falciparum* infection.

There are obvious limitations to in vitro work; the culture well affords an artificial environment that does not parallel the dynamic exchange of cells, cytokines and other immune mediators found in a living organism. However, given the unique receptor repertoire of human NK cells, it would be impossible to delve into the question at hand using a model system such as the mouse, due to the absence of KIR genes in rodents. Hopefully, with the development of more specific and reliable antibody reagents, levels of KIR expression can also be better defined. An alternative method might also be to use quantitative RT-PCR to measure KIR mRNA in responding and nonresponding cells.

In summary, the work presented in this thesis has succeeded in identifying NK cells as an important component of the innate immune response to malaria, and the experiments described have begun to reveal the mechanisms of activation and parasite recognition. As the behavior of these cells in the presence of *P. falciparum* becomes better defined, and the NK cell contribution to protection and/or pathology is determined, vaccine as well as drug design can be modified in order to stimulate these cells appropriately and thus to minimize the detrimental effects of infection.

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Innate Immune Response to Malaria: Rapid Induction of IFN- γ from Human NK Cells by Live *Plasmodium falciparum*-Infected Erythrocytes¹

Katerina Artavanis-Tsakonas and Eleanor M. Riley²

To determine the potential contribution of innate immune responses to the early proinflammatory cytokine response to *Plasmodium falciparum* malaria, we have examined the kinetics and cellular sources of IFN- γ production in response to human PBMC activation by intact, infected RBC (iRBC) or freeze-thaw lysates of *P. falciparum* schizonts. Infected erythrocytes induce a more rapid and intense IFN- γ response from malaria-naïve PBMC than do *P. falciparum* schizont lysates correlating with rapid iRBC activation of the CD3⁺CD56⁺ NK cell population to produce IFN- γ . IFN- γ ⁺ NK cells are detectable within 6 h of coculture with iRBC, their numbers peaking at 24 h in most donors. There is marked heterogeneity between donors in magnitude of the NK-IFN- γ response that does not correlate with mitogen- or cytokine-induced NK activation or prior malaria exposure. The NK cell-mediated IFN- γ response is highly IL-12 dependent and appears to be partially IL-18 dependent. Exogenous rIL-12 or rIL-18 did not augment NK cell IFN- γ responses, indicating that production of IL-12 and IL-18 is not the limiting factor explaining differences in NK cell reactivity between donors or between live and dead parasites. These data indicate that NK cells may represent an important early source of IFN- γ , a cytokine that has been implicated in induction of various antiparasitic effector mechanisms. The heterogeneity of this early IFN- γ response between donors suggests a variation in their ability to mount a rapid proinflammatory cytokine response to malaria infection that may, in turn, influence their innate susceptibility to malaria infection, malaria-related morbidity, or death from malaria. *The Journal of Immunology*, 2002, 169: 2956–2963.

Despite more than two decades of intense research, and a number of clinical trials, there is currently no vaccine that reliably protects against blood stage malaria infections. Vaccine-related research has tended to focus on identification of target Ags of protective immunity rather than the nature of antimalarial immune effector mechanisms, and has, inevitably, concentrated on adaptive rather than innate immune responses. The innate response to malaria has, until recently, received relatively little attention. However, studies in both mice and humans have repeatedly shown that proinflammatory cytokines, specifically IL-12, IFN- γ , and TNF- α , are essential mediators of protective immunity to erythrocytic malaria (1, 2); these cytokines can derive from either the innate or adaptive arm of the immune response. Given that these cytokines play a role in both immunity and pathology of malaria (1, 2), it is important to quantify the relative contribution of these two components of the immune response to the proinflammatory response.

Resistance to rodent malaria is absolutely dependent on signals mediated by IFN- γ (1), and the difference between lethal and non-lethal infections depends on the ability of the mouse to mount an early IL-12, IFN- γ , or TNF- α response (2–5). TNF- α and IFN- γ act synergistically to optimize NO production (6), which is in-

involved in parasite killing (7). Similarly, in humans, IFN- γ production is correlated with resistance to reinfection with *Plasmodium falciparum* (8, 9) and protection from clinical attacks of malaria (10), plasma TNF- α and nitrogen oxide levels are associated with resolution of fever and parasite clearance (11, 12), and plasma TNF- α and IFN- γ mediate loss of infectivity of circulating gametocytes (13). Many vaccine developers now regard IFN- γ production to be the hallmark of effector T cell function for malaria (14, 15).

A recent critical review of the literature (16) concluded that control of the early peak of parasitemia in murine malaria infections was dependent on innate rather than adaptive cellular immune mechanisms, raising important questions about the role of innate immunity in control of human malaria. Enhanced NK cell activity in spleens of mice infected with irradiated *Plasmodium berghei* sporozoites was demonstrated many years ago (17); more recently, *Plasmodium yoelii* sporozoite infection has been shown to induce a rapid inflammatory response in the liver characterized by NK cell, macrophage, and T cell infiltration and IFN- γ production (18). It has been proposed that protective immunity to *P. yoelii* liver stages mediated by parasite-specific CD8⁺ T cells is dependent on the presence of IL-12 and NK cells (19), indicating an important synergy between innate and adaptive immunity in this system. There is less information regarding the role of innate immune mechanisms in controlling blood stage malaria infections; however, depletion of NK cells from *Plasmodium chabaudi*-infected mice results in a more severe course of infection with higher parasitemia and increased mortality (20).

Regarding the human immune response to malaria, we and others have shown that PBMC from malaria-unexposed donors can produce IFN- γ in response to stimulation by either live or dead schizont Ags (21–24). Live parasites induce proliferation of both $\alpha\beta$ and $\gamma\delta$ TCR⁺ T cells, whereas dead schizont extract activates only TCR $\alpha\beta$ ⁺ T cells (24). These cells have been widely assumed

Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom

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² Address correspondence and reprint requests to Dr. Eleanor M. Riley, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K. E-mail address: eleanor.riley@lshtm.ac.uk

to be the major source of IFN- γ (23, 25). Activation of TCR $\alpha\beta^+$ T cells has been ascribed to reactivation of a polyclonal population of memory cells primed by exposure to cross-reactive Ags (21, 26, 27), whereas the TCR $\gamma\delta^+$ T cell response is restricted to the V γ 9V δ 2 subset (28, 29), and is induced by small, phosphorylated nonprotein Ags similar to those described for mycobacteria (30). These findings are consistent with cytokine responses to malaria Ags in unexposed donors being derived from cells of the adaptive immune system. However, Scragg et al. (31) have recently reported very early induction of TNF- α , IL-12, and IFN- γ (within 10 h) by live, parasitized erythrocytes, and Hensmann et al. (32) have shown that live parasites induce TNF- α and IFN- γ from V γ 9 $^+$ T cells and TNF- α from CD14 $^+$ monocytes, within 18 h. Cytokine induction is dependent on the presence of both monocytes and lymphocytes (31), indicating that this is not a classical endotoxin-like response as had previously been thought (33). Increased NK-like cytotoxicity has been reported during mild malaria infection (34), but appears to be depressed in children with severe disease (35). Coculture of PBMC with soluble malaria Ags has been reported to increase their cytotoxic activity against an NK-sensitive cell line (36), and CD3 $^-$ CD56 $^+$ NK cells have been reported to lyse schizont-infected erythrocytes (37).

The purpose of this study therefore was to investigate the kinetics and cellular origins of IFN- γ induced by *P. falciparum* malaria in naive and malaria-exposed human blood donors, to determine the contribution of cells of the innate and adaptive immune response to the early inflammatory response. Our observation that CD3 $^-$ CD56 $^+$ NK cells are major contributors to the first wave of IFN- γ production, within 24 h of PBMC coculture with parasitized erythrocytes, led us to investigate the activation requirements for human NK cells responding to malaria parasites.

Materials and Methods

P. falciparum culture and Ag preparation

P. falciparum parasites of the 3D7 strain were grown in A $^+$ human erythrocytes in RPMI 1640 (Life Technologies, Paisley, U.K.) with 25 mM HEPES (Sigma, Poole, U.K.), 1 μ g/L hypoxanthine (Life Technologies), 28 mM sodium bicarbonate, and 10% normal AB human serum (pH 7.3). Flasks were gassed with 3% O $_2$ /4% CO $_2$ /93% N $_2$ and incubated at 37°C; parasite development was monitored by examination of Giemsa-stained thin blood smears. Cultures were routinely screened for mycoplasma contamination by PCR (BioWhittaker, Wokingham, U.K.) and shown to be mycoplasma free. Mature schizonts were harvested from cultures of 6–7% parasitemia by centrifugation through 60% Percoll (Sigma-Aldrich, St. Louis, MO) and resuspended in culture medium. Schizonts were either placed directly into culture (live parasite Ag, infected RBC (iRBC) 3) or freeze-thawed twice in liquid nitrogen to produce *P. falciparum* schizont extract (PfSL), which was stored at –70°C until used. Freeze-thawed uninfected erythrocytes (uRBC) were used as controls.

PBMC cultures

Thirty adult blood donors were recruited for the study over the course of several months. Fifteen donors were of European descent, four originated from the Indian subcontinent, and eleven were African. Eighteen of the donors had never been infected with malaria, while the remainder had been infected with malaria on one or more occasions in the past. Details of all donors are shown in Table I. Each donor's NK cells were assayed between two and five times on different days to verify the reproducibility of their IFN- γ production, and results were found to be consistent. Venous blood was collected into sterile, heparinized tubes, and PBMCs were separated by density centrifugation (Lymphoprep; Nycomed, Uppsala, Sweden). PBMCs were resuspended at 1×10^6 cells/ml in RPMI 1640 with 2 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin (all Life Technologies), and 10% human AB serum (Sigma-Aldrich). Cells were aliquoted at 10^6 /well into 24-well flat-bottom tissue culture plates (Nunc, Paisley, U.K.) and incubated with the appropriate Ag at 37°C in an atmo-

sphere of 5% CO $_2$ for 1, 2, 4, or 6 days. In initial experiments, iRBC, PfSL, and uRBC were tested for their ability to induce IFN- γ over a range of concentrations (1×10^5 to 1×10^7 schizonts or RBC per well); the optimal concentration of malaria Ags for induction of IFN- γ from cells of naive donors was found to be 3×10^6 parasites/ 10^6 PBMC (which is equivalent to a parasitemia of 15,000 iRBC/ μ l blood), and this was used for all further assays. Growth medium was used as a negative control and induced similar levels of IFN- γ as uRBC. A mixture of human rIL-12 (rhIL-12) (1 ng/ml), rhIL-18 (40 ng/ml) (PeproTech, London, U.K.), and rhIL-2 (2 ng/ml) (Boehringer Mannheim, Lewes, U.K.), or the mitogen PHA (1 μ g/ml; Difco/BD Biosciences, Oxford, U.K.) was used as positive controls for NK cell activation (PHA was not necessarily expected to act directly on NK cells, but to activate other cells in the PBMC culture, inducing cytokines that would lead to NK activation). To determine the effect of exogenous cytokines on the response to malaria parasites, rIL-12 or rIL-18 were added to cultures at concentrations ranging from 0.1 to 10.0 ng/ml.

Flow cytometry

Brefeldin A (10 μ g/ml; Sigma-Aldrich) was added to cell cultures for the last 3 h of incubation. After the appropriate incubation period, supernatants were collected from each culture and stored at –70°C for cytokine analysis by ELISA. Cells were washed twice with FACS buffer (1 \times PBS, 0.1% Na $_2$ S $_2$ O $_8$, 0.1% BSA), resuspended in FACS buffer at a concentration of 5×10^5 cells/ 100μ l, and stained for 40 min at 4°C with appropriate combinations of labeled Abs. Cells were washed twice with FACS buffer, fixed in 2% paraformaldehyde in PBS for 15 min at room temperature in the dark, and washed once with FACS buffer. Cells were resuspended in 100 μ l permeabilization buffer (1 \times PBS, 1% saponin, 0.1% sodium azide) with anti-cytokine Ab, incubated in the dark at 4°C for 30 min, and washed with FACS buffer. Finally, cells were suspended in 300 μ l FACS buffer and analyzed in a FACScan flow cytometer (BD Biosciences). Data analysis was performed with CellQuest software (BD Biosciences). A total of 100,000 events was collected from each sample. The following mAbs were used: CD3 Tricolor (TRI), CD8 TRI, IgG1 TRI (all Caltag Laboratories, Burlingame, CA); TCR $\alpha\beta$ FITC, TCR $\gamma\delta$ FITC, CD56 FITC, CD4 FITC, IgG1 FITC, IgG2 FITC, V γ 9 PE, IFN- γ PE, TNF- α PE, and IgG1 PE (all BD Biosciences); V δ 2 FITC and V γ 9 FITC (Serotec, Oxford, U.K.).

Cytokine ELISA

IFN- γ and IL-12 (p40 and p70) were detected in cell supernatants by sandwich ELISA using commercially available reagents; all samples were tested in duplicate according to the manufacturer's recommendations. IFN- γ Abs and standard were purchased from BD Biosciences; IL-12 Abs and standard were from R&D Systems (Abingdon, U.K.). Where samples gave values above the top of the standard curve, supernatants were retested at 1/10 or 1/100 dilutions in RPMI 1640, and cytokine levels were recalculated.

IL-12 and IL-18 neutralization

Neutralizing goat anti-human IL-12 polyclonal Ab (R&D Systems) was added to PBMC cultures at concentrations ranging from 0.5 to 10 μ g/ml. An isotype-matched control Ab (goat IgG; Sigma-Aldrich) was used at the same concentrations. Neutralizing mouse polyclonal anti-human IL-18 or a mouse IgG1 control Ab (both R&D Systems) were used at concentrations from 0.1 to 5.0 μ g/ml.

Results

IFN- γ response to *P. falciparum* by cells from malaria-naïve donors

We examined the kinetics of IFN- γ secretion from PBMC of five European, malaria-naïve donors in response to iRBC or PfSL, measuring IFN- γ concentration in culture supernatants by ELISA (Fig. 1). uRBC induced minimal IFN- γ production over a period of 6 days. Lysed parasites induced modest, but statistically significant IFN- γ responses in cells from all donors. Cells from three donors produced IFN- γ from about day 4; cells from one donor made only a modest and transient response; and cells from one other donor made high levels of IFN- γ within 24 h. In contrast, live parasites rapidly induced high levels of IFN- γ production from cells from all donors, with IFN- γ concentrations in excess of 10,000 pg/ml by

³ Abbreviations used in this paper: iRBC, infected RBC; PfSL, *P. falciparum* schizont lysate; rh, recombinant human; TRI, Tricolor; uRBC, uninfected RBC.

Table 1. Rapid IFN- γ induction in NK cells by *P. falciparum*^a

	Donor	Ethnicity	Total			NK	
			uRBC	PfSL	iRBC	PfSL (%)	iRBC (%)
Malaria unexposed	053M	E	333	2503	4333	1650 (65.9)	2940 (67.9)
	TH	E	1298	1987	4369	568 (28.6)	1736 (39.7)
	094M	As	1318	845	3175	6 (14.1)	1515 (47.7)
	016F	E	125	385	1634	116 (30.1)	1017 (62.2)
	JR	E	841	971	1940	109 (11.2)	934 (48.1)
	095F	E	4329	4944	5179	637 (12.9)	628 (12.1)
	SB	As	673	679	1136	202 (29.7)	561 (49.4)
	073F	E	749	609	1278	79 (13.0)	470 (36.8)
	PC	E	601	1807	1509	221 (12.2)	260 (17.2)
	014F	E	1222	1315	1378	522 (39.7)	240 (17.4)
	CS	E	608	494	900	34 (6.9)	186 (20.7)
	093F	E	760	932	981	55 (5.9)	148 (15.1)
	AJH	E	2256	3037	2361	183 (6.0)	125 (5.3)
	RaT	As	1216	995	1217	70 (7.0)	77 (6.3)
	KAT	E	282	458	282	61 (12.0)	58 (11.7)
	MH	E	632	534	577	56 (10.5)	54 (9.4)
	BdS	As	612	637	612	30 (4.7)	40 (6.5)
	AMSP	Af	474	544	495	5 (1.0)	19 (3.8)
	Mean \pm SE		1018 \pm 227	1315 \pm 278	1853 \pm 344	256 \pm 94	612 \pm 183
	JET	E	1245	1963	3343	830 (42.3)	1056 (31.6)
Malaria exposed	FMO	Af	556	773	1112	185 (23.9)	537 (48.3)
	HTD5	Af	907	919	1061	118 (12.8)	250 (23.6)
	HTD6	Af	1222	1373	1264	158 (11.5)	149 (11.8)
	HTD3	Af	846	520	1017	45 (8.7)	118 (11.6)
	HT	Af	654	772	807	144 (18.7)	113 (14.0)
	KT	Af	524	615	660	113 (18.4)	103 (15.6)
	HTD1	Af	400	485	348	44 (9.1)	95 (27.3)
	HTD7	E	636	755	1264	84 (1.1)	87 (6.9)
	HTD9	Af	376	356	425	51 (14.3)	67 (15.8)
	HTD8	Af	302	498	291	77 (15.5)	32 (11.0)
	HTD4	Af	540	411	95	30 (7.3)	8 (8.4)
	Mean \pm SE		684 \pm 90	786 \pm 133	973 \pm 243	156 \pm 63	217 \pm 86

^a The early (24 h) IFN- γ response to *P. falciparum*. IFN- γ ⁺ cells were detected by flow cytometry after 24-h coculture of PBMC with malaria-infected iRBC or uRBC or PfSL. Shown are the total number of IFN- γ ⁺ cells/100,000 events and the number of CD3⁺CD56⁺IFN- γ ⁺ cells. Percentages refer to the proportion of IFN- γ ⁺ cells that are NK cells.

day 4 and concentrations of up to 200,000 pg/ml by day 6. Differences between PfSL-induced and iRBC-induced IFN- γ concentrations were statistically significant at all time points (Wilcoxon signed rank test, $Z = -2.023$, $n = 5$, $p = 0.043$). Thus, the IFN- γ response to iRBC was more rapid, and maximal concentrations were 10-fold higher than the response to PfSL.

Cellular source of early IFN- γ production

We hypothesized that the rapid IFN- γ response to iRBC represented triggering of a population of innately activated cells such as NK cells, NK-T, or $\gamma\delta$ T cells. To determine the source of IFN- γ production during the first 3 days of exposure of naive PBMC to malaria Ags, we incubated PBMC with iRBC, PfSL, or uRBC and harvested cells after periods of between 3 and 72 h for analysis of cell surface phenotype and intracellular IFN- γ by flow cytometry. IFN- γ ⁺ cells were gated and analyzed for expression of CD3, TCR $\alpha\beta$, TCR $\gamma\delta$, and CD56. As an example, data for one donor after 24-h incubation of PBMC with 3×10^6 iRBC or uRBC or 3×10^6 lysed infected erythrocytes are shown in Fig. 2. For cells incubated with iRBC, IFN- γ was derived from a mixed population of cells, of which the most numerous are CD56⁺CD3⁺ NK cells. After subtraction of the background counts for the isotype control Ab and uRBC control, 62% of the iRBC-induced IFN- γ ⁺ cells were CD3⁺CD56⁺ NK cells; the remainder were CD3⁺ T cells. After 24-h incubation with PfSL, the number of cells staining for IFN- γ was almost 4-fold lower than for iRBC; after subtraction of background staining, 42% of the IFN- γ ⁺ cells were found to be NK cells.

To determine the optimum parasite concentration for NK cell activation, a dose-response analysis was conducted (Fig. 3, *a* and *b*). Optimal NK cell IFN- γ production was observed at either 1×10^6 or 1×10^7 iRBC/ 10^6 PBMC. For additional experiments, intact and lysed parasites were used at a final concentration of 3×10^6 parasites/ 10^6 PBMC in a volume of 1 ml; control cultures contained 3×10^6 uRBC.

To determine the kinetics of the NK cell response to malaria parasites, the proportion of NK cells making IFN- γ was examined over a period of 72 h (data from two representative donors are shown in Fig. 3, *c* and *d*). iRBC-induced IFN- γ was detected within NK cells as early as 6 h and peaked between 15 and 24 h in all donors. The kinetics of the iRBC-induced response was similar to that of PHA-mediated NK activation. Twenty-four-hour cultures were thus used to detect NK cell IFN- γ production in all future assays.

As others (31, 32) have reported early production of TNF- α from PBMCs and as NK cells have been reported to make TNF- α (38), we also stained NK cells for intracellular TNF- α . As can be seen in Fig. 3, *e* and *f*, a small percentage of NK cells could be induced to express TNF- α after incubation with PHA for 15–24 h, but few, if any, NK cells were induced to secrete TNF- α by incubation with iRBC (note the difference in the scale on the y-axis for IFN- γ and TNF- α).

To determine whether rapid induction of IFN- γ from NK cells by iRBC was a universal phenomenon, we looked at the 24-h IFN- γ response in a cohort of 30 human blood donors (Table 1). The number of IFN- γ ⁺ cells/100,000 events was calculated for

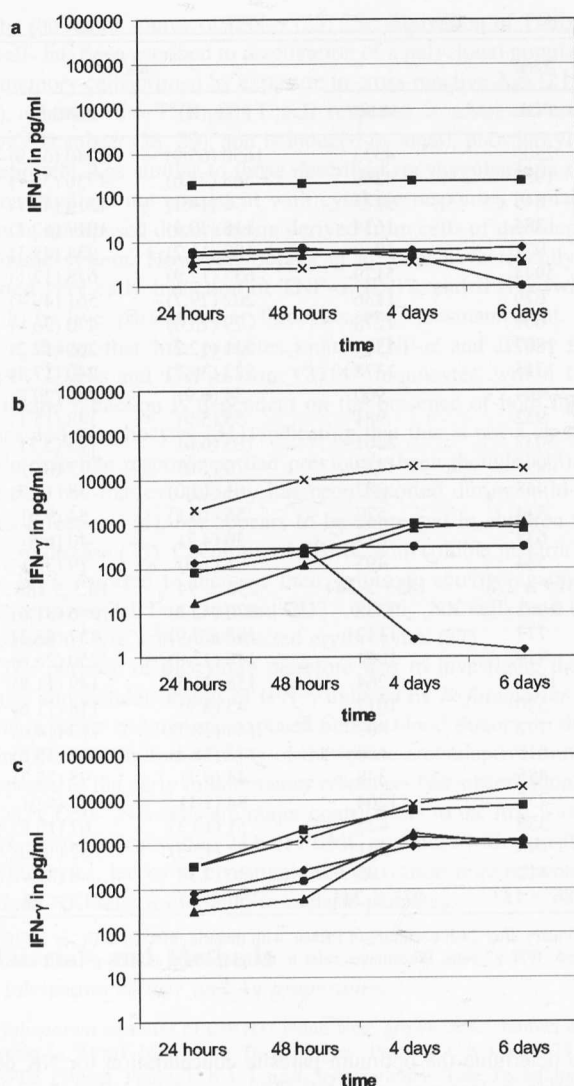


FIGURE 1. Kinetics of IFN- γ (pg/ml) production from PBMCs of malaria-naïve blood donors. PBMCs (10^6 /ml) were cultured with 3×10^6 uRBC (a), 3×10^6 freeze-thawed schizonts (PfSL) (b), or 3×10^6 live, parasitized RBC (iRBC) (c) for up to 6 days. Cell supernatants were collected at intervals and assayed for IFN- γ by ELISA. Values for uRBC-stimulated cultures have been subtracted from values for iRBC- and PfSL-stimulated cultures. Data from five donors are shown, each donor represented by a different symbol.

PBMC cultured with uRBC, iRBC, or PfSL, and the number (%) of the IFN- γ^+ cells that were NK cells (i.e., CD3 $^-$, CD56 $^+$, IFN- γ^+) is also shown. In both the malaria-unexposed and malaria-exposed donors, there is marked heterogeneity in the number of IFN- γ^+ cells in both PfSL and iRBC cultures. In the malaria-unexposed donors, iRBC induced significantly higher numbers of PBMC to produce IFN- γ than did PfSL (Wilcoxon signed rank test, $Z = 2.308$, $p = 0.021$), but this difference was not significant for the malaria-exposed donors ($Z = 1.177$, $p = 0.239$). Similarly, in unexposed donors, iRBC induced significantly more NK cells (in terms of both absolute numbers and percentages) to produce IFN- γ than did PfSL ($Z = 2.591$, $p = 0.009$) and, again, this difference was not significant for the malaria-exposed donors ($Z = 1.255$, $p = 0.209$). The total number of IFN- γ^+ cells, and particularly the number of IFN- γ^+ NK cells, was somewhat lower in the malaria-exposed donors than in the malaria-unexposed donors, but there was considerable heterogeneity within the malaria-exposed

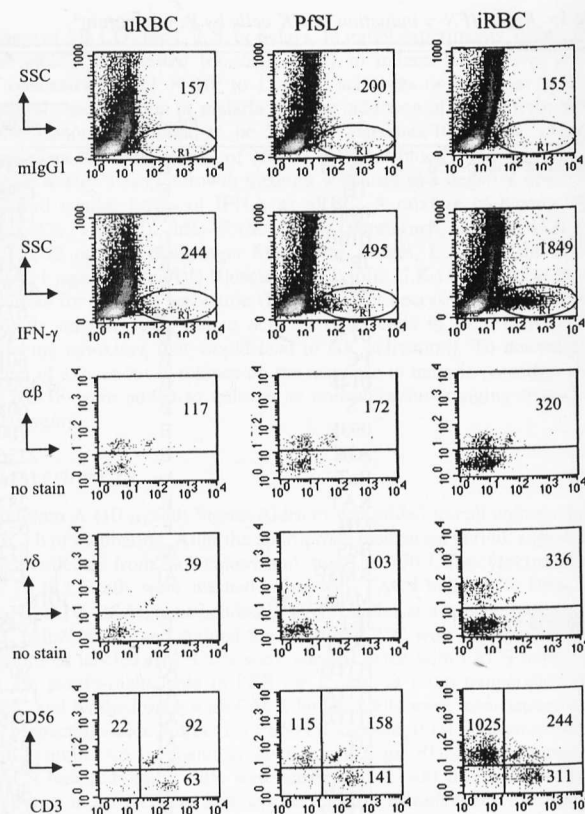


FIGURE 2. Cellular source of IFN- γ after 24 h in vitro activation of PBMC with malaria Ags. PBMCs (10^6 /ml) were cultured with 3×10^6 uRBC, 3×10^6 freeze-thawed schizonts (PfSL), or 3×10^6 live, parasitized RBC (iRBC) for 24 h. Intracellular IFN- γ and cell surface phenotype were analyzed by flow cytometry. Isotype control data are shown in the top row. IFN- γ^+ cells were gated (second row) and analyzed for expression of TCR $\alpha\beta$ (third row), TCR $\gamma\delta$ (fourth row), and CD56/CD3 (bottom row). The number of IFN- γ^+ cells/100,000 events was counted.

group and these differences were not statistically significant (Student's t test, $t \leq 1.88$, $p \geq 0.07$ for all comparisons). Perhaps the most noticeable trend in the data was the marked variation between donors in the numbers of IFN- γ^+ NK cells that were induced by iRBC (ranging from 2,940 cells/100,000 events to less than 20).

Taken together, these data suggest that: 1) the ability to mount a rapid IFN- γ response to malaria parasites is partially dependent upon the ability of NK cells to respond to malaria parasites; 2) the difference in the magnitude of the response to iRBC and PfSL is due in large part to the ability of iRBC to activate NK cells; and 3) individuals vary in their ability to make an NK cell response to malaria parasites.

Heterogeneity of the human NK cell response to malaria parasites

Variation between individuals in their T cell response to specific Ags is commonplace and relatively easily explained by differences in T cell repertoire, MHC genotype, and prior exposure to Ag. Substantial variation in the NK cell response to a given stimulus was less expected and less easy to explain, as this represents an innate response in which prior exposure to the pathogen is not expected to augment the response. Indeed, NK cells from malaria-exposed donors were somewhat less likely to make IFN- γ than cells from naïve donors (Table I and Fig. 4). One possibility is that the heterogeneity represents inherent differences between donors in the ease with which their NK cells are activated to produce

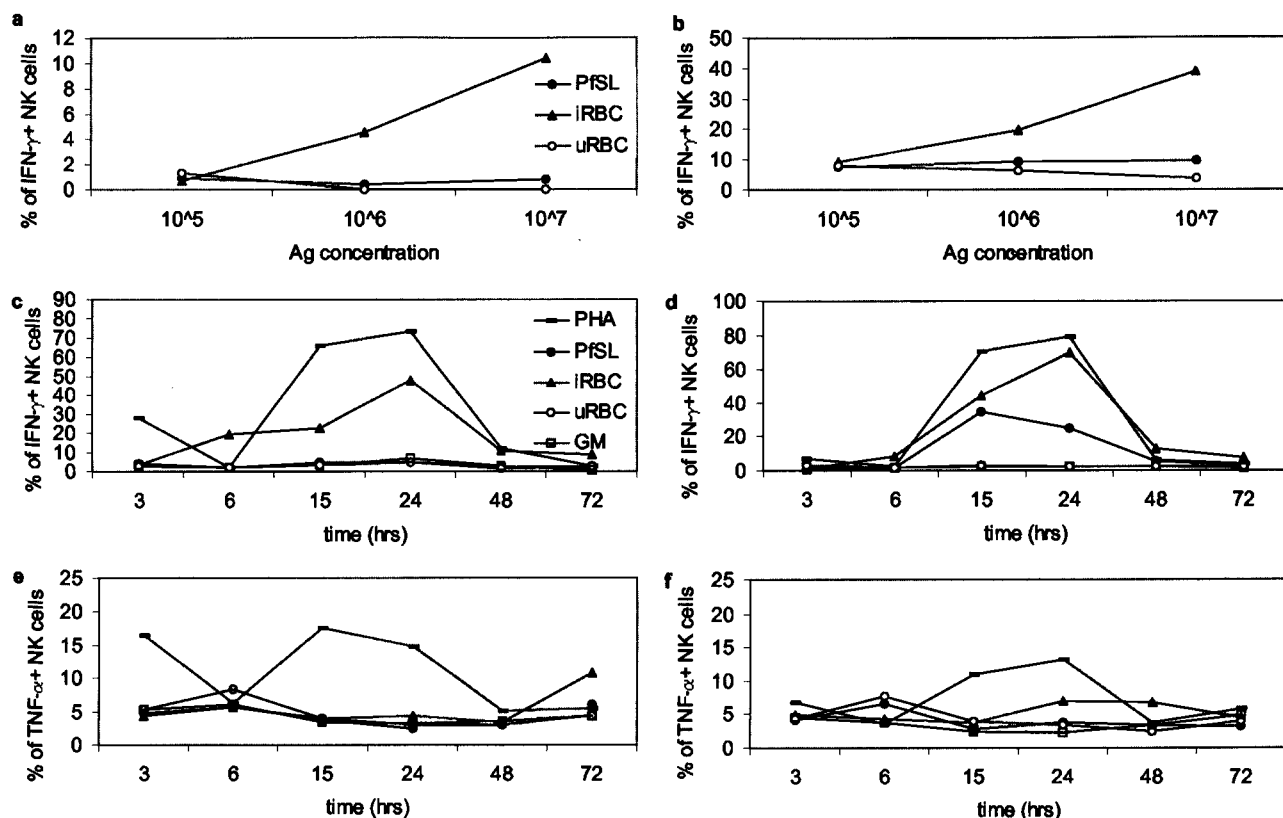


FIGURE 3. Dose response and kinetics of cytokine production by CD56⁺CD3⁻ NK cells. PBMC (10⁶/ml) were incubated with iRBC, PfSL, or uRBC (3 × 10⁶/ml) and stained for flow cytometric analysis. CD56⁺ cells were selected by gating on side scatter and CD56⁺; the gated cells were then analyzed for IFN-γ and CD3 expression. The proportion of CD3⁻CD56⁺ cells that were positive for IFN-γ is shown; data are based on collection of 100,000 events. The effect of parasite dose (a and b) was determined by addition of increasing concentrations of cells or parasite lysate to cultures. The kinetics of the IFN-γ (c and d) or TNF-α (e and f) response was determined by culturing cells with iRBC, PfSL, uRBC, or PHA (1 μg/ml) for up to 72 h.

IFN-γ. However, we found no obvious correlation between the response to iRBC and the response to other stimuli such as recombinant cytokines or (as shown in Fig. 4) PHA.

Is NK cell activation dependent on IL-12 or IL-18?

As activation of NK cells is known to be at least partially IL-12 and IL-18 dependent in many systems (39–41), we hypothesized that the difference between donors in their ability to make an NK cell response to malaria might be due to differences in their ability to make IL-12 or IL-18 in response to iRBC activation.

To determine whether NK cell responses to malaria were indeed IL-12 or IL-18 dependent, we incubated PBMC with iRBC or uRBC for 24 h in the presence or absence of increasing concentrations of neutralizing Ab to human IL-12 or IL-18 (Fig. 5). The percentage of IFN-γ⁺ NK cells was markedly reduced in the presence of 0.5 μg/ml anti-human IL-12, but not by equivalent concentrations of an isotype-matched Ab (Fig. 5a); NK cell responses were not further reduced by increasing doses of Ab. Anti-IL-12 at a concentration of 0.5 μg/ml consistently reduced the percentage of IFN-γ⁺ NK cells by between 50 and 100% (Fig. 5b). In a comparable experiment, anti-IL-18 Ab had rather variable effects on the proportion of NK cells that made IFN-γ in response to iRBC. In titration experiments, anti-IL-18 Ab inhibited NK activation by up to 50% at high concentrations (5.0 μg/ml) (Fig. 5c) and was able to partially inhibit induction of IFN-γ in NK cells of some donors (e.g., 094M and FMO), but not others. (e.g., 053M) (Fig. 5d).

As the NK cell response to iRBC was clearly IL-12 dependent, we wondered whether the difference between high and low re-

sponder donors was due to differences in IL-12 induction from macrophages or dendritic cells in the PBMC culture. Absolute levels of IL-12 p40 or p70 in culture supernatants at 24 h were very low (frequently undetectable) and did not differ between antigenic stimuli or between donors (data not shown). More importantly, addition of rIL-12 at concentrations from 0.1 to 10.0 ng/ml to PBMC cultured with either PfSL or iRBC had no consistent effect on either the concentration of IFN-γ in supernatants or the percentage of IFN-γ⁺ NK cells after 24 h (data not shown). Also, addition of exogenous rIL-12 did not enhance the response of low responding donors and did not enhance PfSL-induced responses to the level of iRBC-induced responses (data not shown). Thus, although IL-12 plays an essential role in the activation of NK cells to make IFN-γ, lack of IL-12 is insufficient to explain the differences in IFN-γ responses between individuals or between live and dead parasites.

In a comparable experiment, addition of rIL-18 to 24-h PBMC cultures with PfSL or iRBC had no effect on the NK cell response to PfSL, although low doses of rIL-18 (0.1 or 1.0 ng/ml) did marginally enhance the NK response to iRBC in most donors (data not shown).

Discussion

The main aim of immunological research on malaria over the past 20 years has been vaccine development and has thus, by necessity, focused on adaptive immune responses. The innate response to malaria has received relatively little attention. However, growing awareness of the protective role of innate immune mechanisms in their own right and their role in induction of adaptive immunity

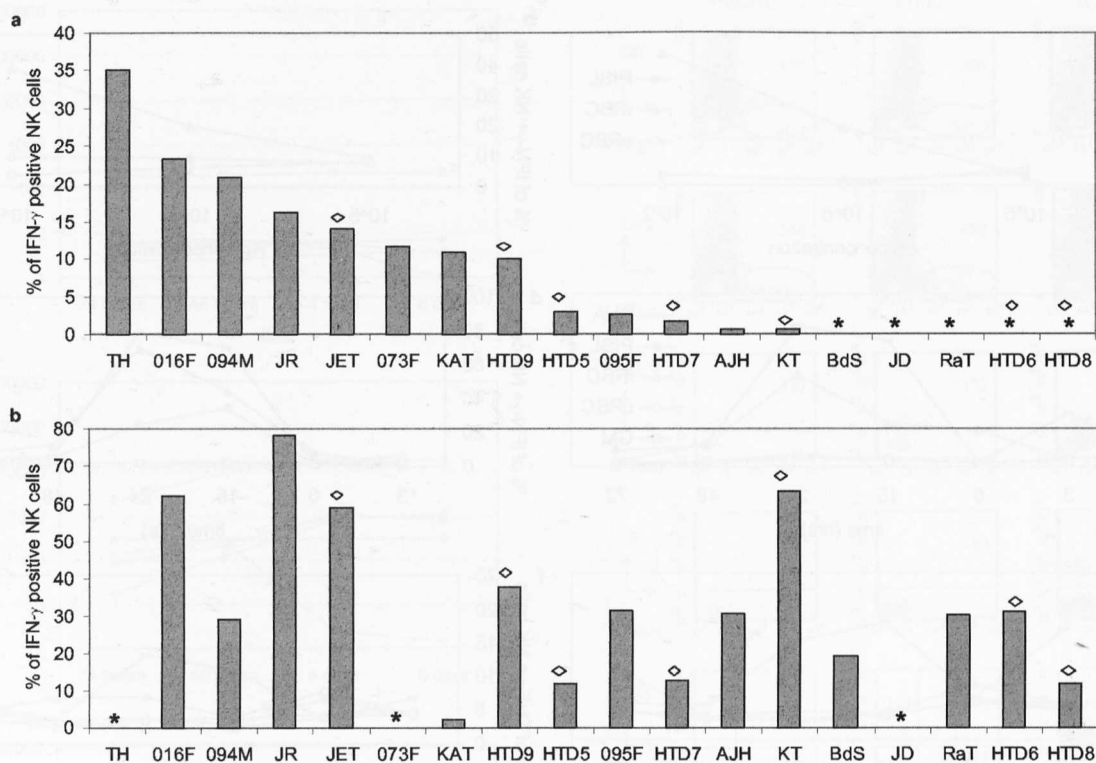


FIGURE 4. Heterogeneity of the human NK cell response to iRBC. The percentage of NK ($CD3^+CD56^+$) cells staining for intracellular IFN- γ after 24-h coculture with iRBC (uRBC values subtracted) (a) or PHA (growth medium values subtracted) (b) is shown. $CD56^+$ cells were selected by gating on side scatter and $CD56^+$; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportion of $CD3^+CD56^+$ cells that were positive for IFN- γ is shown; data are based on collection of 100,000 events. Data from 18 individual donors are shown. All donors are malaria naive except for those marked \diamond who have varying levels of prior malaria exposure. *, No IFN- γ^+ NK cells detectable. Comparison of NK responses to iRBC and a recombinant cytokine (IL-2/12/18) mixture ($n = 11$) showed a similar lack of correlation (data not shown).

suggests that studies of innate immunity to malaria are warranted. Rapid induction of monokines and lymphokines (such as IL-12, IFN- γ , and TNF- α) may enable the infected host effectively to control the exponential replication of blood stage parasites until the adaptive immune response can take over. This might be of most benefit during a primary infection, but, given the extent of antigenic polymorphism in malaria, innate responses may also be required to control reinfections of variant genotype until novel adaptive responses can be generated.

It has long been known that T cells from malaria-naïve donors can proliferate and secrete cytokines in response to *P. falciparum* Ags. Responses tend to peak after 6–7 days activation in vitro and the proliferating cells have been identified as either $TCR\alpha\beta^+$ T cells, which respond in a classical MHC-restricted manner to both live and dead parasite Ags, or $TCR\gamma\delta^+$ T cells that respond preferentially to live parasites (23, 24); both cell types have also been shown to secrete IFN- γ (21, 23, 25). Recently, it has been suggested that live *P. falciparum* can induce rapid (within 12–24 h) IFN- γ and TNF- α responses (31) and that $TCR\gamma\delta^+$ T cells may contribute to the early IFN- γ response (32). We have also recently demonstrated an important role for IL-12 in malaria-induced IFN- γ production (42). We have therefore conducted a detailed study of the kinetics of IFN- γ production by *P. falciparum*-activated PBMC.

The first interesting observation was that live parasites induce a much stronger and much more rapid IFN- γ response than do dead parasites. Differences between live and dead parasites have been noticed previously, particularly in their ability to activate $TCR\gamma\delta^+$ T cells (24, 32). We have now extended this observation to include preferential induction of IFN- γ production in NK cells by live parasitized erythrocytes. The requirement for live parasites within

intact RBC for induction of the innate response may be due to a need for direct contact between the parasitized red cell and the leukocyte (be it a lymphocyte or an accessory cell), or may be due to instability of the parasite-derived ligands that interact with cell surface receptors.

The nature of the malarial ligands that might be involved in induction of the innate response is largely unknown. Scragg et al. (31) have shown that early cytokine induction by *P. falciparum* is not due to the presence of classical endotoxins: levels of IL-12 and IL-10 induced by malaria Ags are orders of magnitude lower than the levels induced by binding of bacterial LPS to CD14, and a $CD14^+$ monocyte-like cell line that responds to a wide variety of bacterial endotoxins by secretion of TNF- α fails to respond to *P. falciparum* (31). Malarial GPIs have been shown to activate macrophages and vascular endothelium (33, 43). GPIs from *Trypanosoma cruzi* have been shown to induce IL-12 via binding to Toll-like receptor 2 (44), but nothing is currently known of malarial ligands for Toll-like receptors. *P. falciparum*-derived, phosphorylated, nonprotein moieties similar to those isolated from mycobacteria have been shown to be ligands for $TCR\gamma\delta^+$ T cells (30) and, due to their highly lipophilic nature, are likely to have a rather short $t_{1/2}$ in solution. Finally, malarial GPIs have also been reported to activate murine CD1d-restricted NK-T cells (45), although these cells then produce IL-4 rather than IFN- γ , but at least one study has failed to reproduce the findings (46).

In this study, we have shown that $CD3^+CD56^+$ NK cells are major contributors to the early IFN- γ response to malaria parasites. IFN- γ^+ NK cells can be identified as early as 6 h after stimulation in some donors, and the peak of the NK IFN- γ response occurs between 15 and 24 h. Preliminary studies in our laboratory indicate

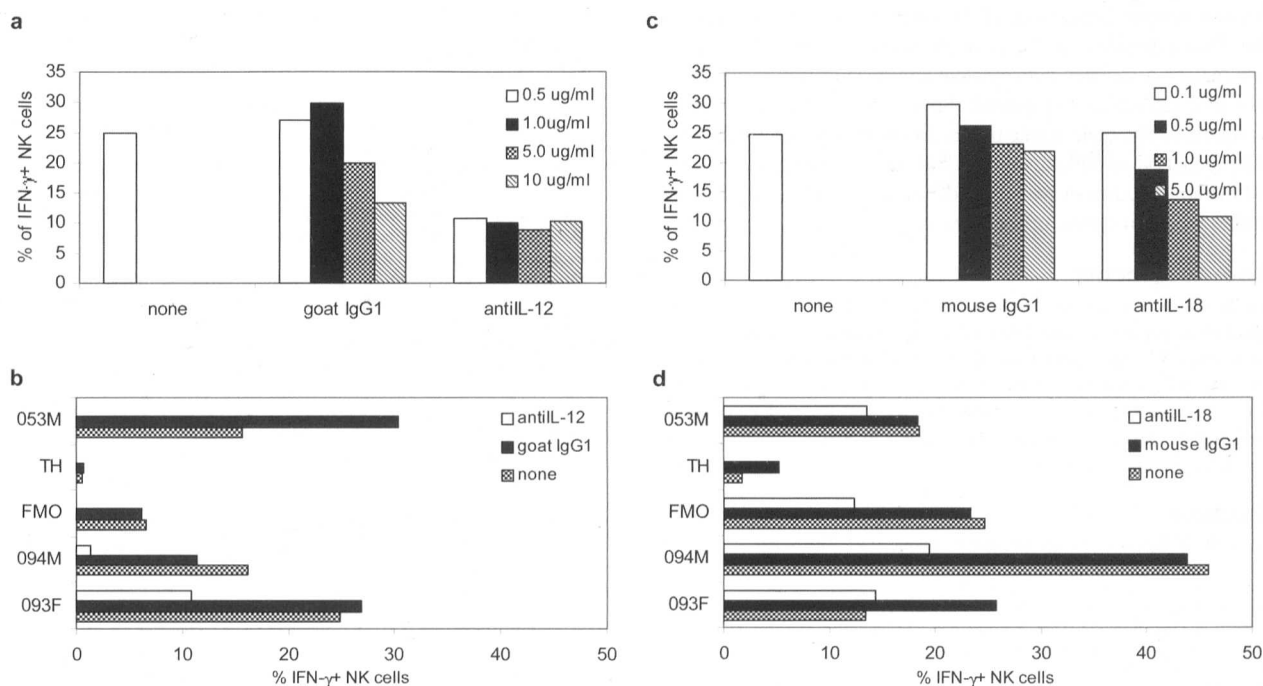


FIGURE 5. Effect of neutralizing Ab to IL-12 and IL-18 on IFN- γ production from NK cells after 24-h coculture with iRBC. PBMCs (10^6 /ml) were cultured with 3×10^6 iRBC, or 3×10^6 freeze-thawed schizonts (PfSL) for 24 h in the presence of neutralizing Ab to human IL-12 (a and b) or IL-18 (c and d) or control, isotype-matched Abs. Control cultures were incubated with 3×10^6 uRBC; uRBC values have been subtracted from iRBC and PfSL values. CD56 $^{+}$ cells were selected by gating on side scatter and CD56 $^{+}$; the gated cells were then analyzed for IFN- γ and CD3 expression. The proportion of CD3 $^{-}$ CD56 $^{+}$ cells that were positive for IFN- γ is shown; data are based on collection of 100,000 events. Optimal anti-cytokine Ab concentrations were determined by titration (a and c); data for five donors at this optimal concentration are shown (b and d).

that the activated NK cells undergo apoptosis from ~24 h (K. Artavanis-Tsakonas and E. Riley, unpublished observation). In the majority of donors, NK cells are the first population to become IFN- γ^{+} , with $\gamma\delta$ T cells becoming IFN- γ^{+} after 48–72 h and $\alpha\beta$ T cells beginning to make IFN- γ after 4–6 days (data not shown).

We were not surprised to find that the NK cell response was highly IL-12 dependent; this is widely reported for NK cells (39), and has been shown for NK responses to a number of pathogens (40, 41). We considered the possibility that differences between donors, and between Ag preparations, in the magnitude of the IFN- γ response might be due to differences in IL-12 induction. However, addition of rIL-12 did not significantly enhance the response of individual donors and did not increase the response to PfSL to levels seen for iRBC.

The requirement for IL-18 was less clear, with some donors showing a reduction in NK cell responses to iRBC in the presence of anti-IL-18 Ab and cells from other donors being unaffected. In contrast, low doses of rIL-18 did marginally enhance the proportion of NK cells that could be induced to produce IFN- γ in response to iRBC in most donors, indicating that IL-18 can indeed augment the NK response to malaria parasites. Taking the IL-12 and IL-18 data together, it appears that while monokines, produced for example by dendritic cells or macrophages in the PBMC cultures, are required to optimize the NK cell response to *P. falciparum*, these monokines are not sufficient on their own to induce the levels of IFN- γ production obtained with malaria-infected RBC. A lack of IL-12 or IL-18 in the culture medium is insufficient to explain the very low levels of NK cell activation induced by parasite lysates or the very low responses to iRBC in some donors. However, the influence of other cytokines (e.g., IL-2 and IL-15) and the ability of an individual's macrophages or dendritic cells to respond to malaria Ags in other ways require further investigation.

In addition to NK activation by cytokines, our data raise the possibility that ligands expressed on the intact iRBC are specifically recognized, either by the NK cell itself or by an APC population. Preliminary data from our laboratory indicate that iRBC can bind to NK cells and that contact between the NK cell and the iRBC is required for optimal IFN- γ induction (K. Artavanis-Tsakonas and E. Riley, unpublished data). Also, there is a report in the literature that human NK cells can lyse parasite-infected RBC (37), indicating that direct cell:cell contact does occur. Thus, optimal activation of NK cells may require integration of two or more signals transduced through different receptors.

It is clear that there is considerable variation between individuals in the magnitude of their NK cell IFN- γ response. This heterogeneity does not appear to be due to inherent differences in the ease of NK activation or differences in IL-12 or IL-18 production. The difference between malaria-exposed and malaria-unexposed donors is intriguing. Although the numbers are quite small, and the difference not quite statistically significant ($t = 1.878$, $p = 0.07$), the numbers of NK cells making IFN- γ were lower in iRBC-stimulated cultures from the exposed than the unexposed donors. At this stage, we cannot say whether this represents down-regulation of NK cell responses by acquired immune responses or is due to genetic differences between the groups (10 of the 12 exposed donors were Africans compared with only 1 of 18 in the unexposed group). A possibility is that genetic variation in NK cell receptor expression, particularly of activating killer cell Ig-like receptors (47), might influence the response to malaria.

Importantly, our data suggest that the NK cells are a significant source of IFN- γ in the first few hours of a malaria infection, and thus may be an important component of the innate defense against malarial parasitemia. Surprisingly, this innate response is not universal among human blood donors, raising interesting questions

about the functional importance of the innate response to malaria infection. Two opposing hypotheses can be proposed; rapid IFN- γ production may be associated with efficient induction of IFN- γ -mediated effector mechanisms and enhanced ability to control malaria infections. Alternatively, rapid innate production of IFN- γ may predispose to overproduction of inflammatory cytokines and increased risk of severe malaria. Studies in malaria endemic areas will be required to determine which, if either, of these scenarios is correct.

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